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(54) Title: DEFECTIVE HEPADNAVIRUSES AND PRODUCER CELL LINE FOR VACCINES AND TREATMENT OF LIVER DISEASES AND DISORDERS

(57) Abstract

The present invention relates to replication-defective hepadnaviruses. In particular, the invention relates to two types of defective hepadnavirus genomes, and the nucleic acid sequences thereof. The first type (termed herein "particle-defective"genomes) are incapable of supplying all hepadnaviral functions required for replication, but are able to produce a pregenomic RNA with the appropriate *cis*-acting signals necessary for inclusion of the RNA in virions (packaging) and for reverse transcription int DNA. The second type of defective hepadnavirus genomes of the invention, termed herein "packaging genomes", produced pregenomic RNA which cannot be packaged and/or reverse-transcribed into a double-stranded genomic DNA, and produce messenger RNAs capable of supplying functions required in *trans* for packaging. The present invention also relates to therapeutic uses of both hepadnavirus packaging genome products and virion particles consisting of packaged particle-defective genomes such as the prevention of hepadnavirus infection or its sequelae and for purposes of gene therapy.

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DEFECTIVE HEPADNAVIRUSES AND PRODUCER CELL LINE FOR VACCINES AND TREATMENT OF LIVER DISEASES AND DISORDERS

1. Field of the Invention

This application is a continuation-in-part of Serial No. 232,811, filed August 16, 1988.

The present invention relates to replication-defective hepadnaviruses. In particular, the invention relates to defective hepadnaviruses that are incapable of replication by themselves, yet whose pregenomic RNA, in the presence of an appropriate helper virus function, can be packaged and reverse transcribed into DNA. In specific embodiments, the genomic DNA of such viruses can have deletions in the env, pol, and/or core genes. The invention also relates to hepadnavirus packaging genomes, whose encoded pregenomic RNA cannot itself be packaged and/or reverse-transcribed into a double stranded DNA genome, yet is capable of supplying functions required in trans for packaging. The defective hepadnaviruses of the invention which express immunogenic epitopes may be formulated as vaccines, or used as immunostimulatory agents for the production of an immune response against hepatitis virus antigens.

The present invention is also directed to defective hepadnaviruses which contain a heterologous gene sequence. In one embodiment of the invention, these recombinant viruses may be used for gene therapy of an inherited deficiency of an hepatic enzyme or an enzyme whose deficiency can be replaced by hepatic production. In another embodiment of the invention, recombinant hepadnaviruses containing a heterologous gene sequence encoding an imunogenic epitope may be formulated as vaccines for protection against infection by a pathogenic organism or for protection against conditions or disorders caused by an antigen of the organism.

The pres nt invention is also directed to the generation and maintenance of permanent hepatic cell lines which are stably transfected with the

defective hepadnaviruses of the invention and are capable of producing infectious defective hepadnavirus particles.

The defective hepadnaviruses of the invention may also be formulated as therapeutic interfering agents, preventing the propagation or maintenance of infection by wild-type virus.

2. BACKGROUND OF THE INVENTION

2.1 HEPADNAVIRUSES

2.1.1. STRUCTURE OF HEPADNAVIRUSES

Hepadnaviruses, which include human hepatitis B virus (HBV) (Barker et al., 1975, Am. J. Med. Sci. 270:189-196), woodchuck hepatitis B virus (WHBV) (Ogston et al., 1982, Cell 29:385-94), duck hepatitis B virus (DHBV) (Summers and Mason, 1982, Cell 29:403-415), and ground squirrel hepatitis B virus (GSHBV) (Marion et al., 1980, Proc. Natl. Acad. Sci. U.S.A. 77:2491-2495) are hepatropic, lead to persistent infection, and have a common structure. Large concentrations of hepadnaviruses have been detected in the blood of infected organisms. Hepadnavirus virions are approximately 42 nm in diameter and consist of an envelope and nucleocapsid (reviewed in Ganem and Varmus, 1987, Ann. Rev. Biochem. 56:651-693 and Tiollais et al., 1985, Nature (London) 317:489-495). The envelope contains the hepatitis B surface antigen (HBsAg) as well as carbohydrates and lipids. The nucleocapsid contains a circular DNA (3.0-3.3 kb in length), a DNA polymerase, protein kinase activity, and hepatitis B core antigen (HBcAg).

The hepadnavirus genome is a small, circular, partly double stranded DNA molecule (reviewed in Ganem and Varmus, supra and Tiollais et al., supra). The

minus strand is linear and of a fixed length, 3-3.2 kb. In contrast, the plus strand is of variable length, ranging from 50-100% of that of the minus strand.

The results from analyses of nucleotide sequences of cloned genomes of hepadnaviruses indicated that the minus strand with the exception of DHBV contains four major open reading frames (ORFs), S, C, P, and X (reviewed in Ganem and Varmus, supra and Tiollais, supra). DHBV contains S, C, and P ORFs (see figure 1). ORF S, which codes for HBsAg, is divided into the S gene, pre-S1 region, and pre-S2 region. Similarly, ORF C, which codes for HBcAg, is divided into the C gene and pre-C region. ORF P encodes the viral polymerase. ORF X can potentially encode a polypeptide 154 amino acids in length. The function of ORF X, though, is unknown.

The replication of hepadnavirus genomes involves four major steps (reviewed in Ganem and Varmus, supra; Mason et al., 1987, Adv. Virus Res. 32:35-96; and Seeger et al., 1986, Nature (London) 232:477-484). The first step involves the conversion of the asymmetric DNA found in virions to covalently closed circular DNA within the nucleus of infected cells. The covalently closed circular DNA is then transcribed by RNA polymerase II to generate two RNA species, genomic and subgenomic. The genomic RNA (3.5 kb) contains the full complement of viral genetic information and can therefore serve as both a replication template and mRNA. The subgenomic RNAs, 2.1 and 2.4 kb in length, are most likely mRNAs for the pre-S1 (2.4 kb transcript) and pre S2 and S proteins (2.1 kb transcript).

Minus strand DNA is then synthesized by reverse transcription of pregenomic RNA using the viral polymerase and a protein primer. The initiation site for minus strand DNA synthesis is thought to ccur within the short sequence, DR1 (direct repeat 1).

The final step, the synthesis of plus stranded DNA is accomplished by copying the minus strand template using virus associated polymerase along with an oligoribonucleotide primer. Both DR1 and DR2 play important roles in this step (further detail is provided in Ganem and Varmus, supra; Seeger et al., 1987, Adv. Virus Res. 32:35-96). An RNA primer is apparently employed, derived from the 5' portion of the pregenomic RNA. This primer contains the DR1 sequence which has been proposed to anneal to the DR2 sequence present in the minus strand (Madson et al., 1987, Adv. Virus Res. 32:35-96) to enable priming of plus strand synthesis. The plus strand synthesis proceeds for variable distances, often terminating before complete copying of the minus strand.

The structure and expression of the hepatitis B viral genome has been studied by recombinant DNA techniques. U.K. Patent Publication No. GB 2034323A (published June 4, 1980) discloses the cloning and expression of HBV, DNA. Dubois et al. (1980, Proc. Natl. Acad. Sci. U.S.A. 77:4549-4553) have reported the expression of HBV surface antigen and excretion of surface antigen particles after cotransformation of mouse cells with cloned HBV DNA and the herpes simplex thymidine kinase gene. Moriarty et al. (1981, Proc. Natl. Acad. Sci. U.S.A. 78:2606-2610) described the construction and expression of a recombinant simian virus containing a fragment of HBV DNA encoding the HBsAg; 22 nm surface antigen particles were shown to be excreted into the cell culture medium. European Patent Publication No. 0020251 (published December 10, 1980) describes the construction and expression of recombinant expression vector encoding HBV proteins. European Patent Publication No. 0013828 (published August 6, 1980) discloses recombinant DNA vectors which contain fragments of HBV DNA, isolated from Dane

particles, which encode proteins with HBV antigenicity.

2.1.2. BIOLOGY OF HEPADNAVIRUS INFECTION

HBV infection has been observed to be highly polymorphic, ranging from inapparent forms in which individuals experience mild or no liver injury to acute hepatitis B, a moderately severe illness characterized by hepatocellular injury and inflammation to severe chronic liver disease (reviewed in Ganem and Varmus, 1987, Ann. Rev. Biochem. 56:651-693). The virus itself appears not to be cytotoxic. Variation in host immune response to virus infected cells appears to be a major determinant in the degree of severity of liver damage in individuals. Both humoral and cellular immune responses to HBcAg and HBsAg has generally been observed during acute and chronic HBV infection (reviewed in Robinson, 1986, In Fundamental Virology, Fields, B.N. and Knipe, D.M. (eds.), Raven Press, N.Y., pp. 657-679). Other hepadnaviruses have been observed to be mainly hepatropic and lead to persistent virus infection. Chronic active hepatitis has been observed to be the result of HBV and WHBV infection.

In addition to infecting hepatocytes, HBV DNA has been detected in such nonhepatic tissues as kidney, pancreas, and skin (Robinson, supra). Free viral DNA has also been detected in the kidney and pancreas of infected Peking ducks. HBV DNA has also been detected, though in lower copy number than in hepatocytes, in peripheral blood leukocytes and the bone marrow cells.

Hepadnavirus DNA in hepatocytes can exist as either free DNA or integrated into the host cellular chromosome (Tiollais et al., 1985, Nature (London) 317:489-495). Free HBV DNA is detected during acute and some chronic stages of HBV infection and usually

represents intermediate forms of replication. In contrast, integrated sequences are mostly observed during chronic virus infection and hepatocellular carcinoma.

In addition to causing acute and chronic liver disease, results from epidemiological and molecular biological studies indicate a connection between hepadnavirus infection and hepatoma (reviewed in Ganem and Varmus, supra; Machowiak, 1987, Am. J. Med. 82:79-97; and Tiollais et al., 1985, Nature (London) 317:489-495. There is a strong correlation between high rates of chronic HBV infection and incidence of hepatoma, e.g., in Southwest Asia and equatorial Africa. In addition, the hepatocytes of hepatoma patients have been found to contain HBV DNA and HBsAg. Hepatoma has also been observed in animals chronically infected with hepadnaviruses. Furthermore, hepatoma was able to be induced experimentally in woodchucks by inoculation with WHBV at birth.

The structure of hepadnavirus DNA sequences present in human (Dejean et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:5350:5354), woodchuck (Ogston et al., 1982, Cell 29:403-415), ground squirrel (Marion et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:4543-4546) and duck (Imazeki et al., 1988, J. Virol. 62:861-862) hepatocellular carcinoma has been studied by Southern blot hybridization. It has generally been observed that viral DNA is integrated into the host chromosomal DNA at 1 to 12 sites. The virus-host chromosome junctions have generally been located in the vicinity of the cohesive end region between DR1 and DR2.

2.1.3. VACCINATION AND THERAPIES FOR HBV INFECTION

Current methods used to treat HBV infection may be divided into two categories, antivirals and immunomodulatory agents (revi wed in Hoofnagle, Ann.

Int. Med. 107:414-415). One analog that has been widely tested is vidarabine (adenine arabinosid) an adenine analog with potent activity against herpesviruses. While the inhibition of viral replication was observed to be nearly complete and sustained in some patients, the inhibition of virus was partial and transient in other patients. One drawback involved in using this drug is that it is highly water insoluble and has to be administered intraveneously as a constant infusion. A monophosphorylated derivative of vidarabine, vidarabine phosphate can be administered by rapid intravenous infusion or intramuscularly. Even though vidarabine phosphate therapy resulted in the clearance of serum hepatitis B virus DNA, it did not lead to a sustained improvement in the accompanying liver disease. Other antivirals that have been tested for their effectiveness in treating HBV infection include acyclovir and suramin.

Studies have also been undertaken in which patients were treated with either lymphoblastoid or recombinant alpha-interferons for 1-6 months. It was found that only 25-40% of the patients responded to this therapy. The combination of vidarabine phosphate and human leukocyte interferon proved to be toxic. Other therapies currently being studied include the administration of interleukin 2, gamma interferon, and short course corticosteroids.

U.S. Patent No. 4,741,901 discloses a vaccine comprising a 22 nm polypeptide particle made of mature hepatitis B surface antigen.

2.2. DEFECTIVE VIRUSES

2.2.1. DEFECTIVE RNA VIRUSES

In most preparati ns of such RNA viruses as vesicular stomatitis virus (VSV), parainfluenza virus,

influenza viruses, alphaviruses and reoviruses (reviewed in Holland, 1986, In Fundamental Virology, Fields, B.N. and Knipe, D.M., eds., Raven Press, NY pp. 77-99); there are numerous particles known as defective interfering (DI) particles, that have genomes which contain only a portion of the genetic information of the parental virus. DI particles are not capable of self-replication in host cells, but do replicate when they coinfect a cell with a homotypic infectious virus (helper virus) that supplies the missing gene products. Replication of helper virus is greatly suppressed (autoinferference) since DI particles complete for limited amounts of RNA polymerase supplied by infectious particles.

DI particles have been found to play a role in both acute and chronic infections. It has been shown that VSV DI particles modulate virulence in mice by initiating a cyclic pattern of VSV growth in vivo (Cave et al., 1985, J. Virol. 55:366-373). In addition, the establishment and maintenance of persistent VSV infection in baby hamster kidney cells also requires DI particles (Holland and Villareal, 1974, Proc. Natl. Adad. Sci. U.S.A. 71:2956-2960).

The origin of DI particles may lie in aberrant replication events (Lazzarini et al., 1981, Cell 26:145-154). It is postulated that in these events the polymerase-nascent strand complex detaches from the template and replication is completed on another template or at a different position on the same template.

2.2.2. DEFECTIVE RETROVIRUSES

Defective retroviruses primarily differ from other RNA defective viruses in that defective retroviruses generally do not modulate infection of full length retrovirus particles. However, defective retrovirus s like th r def ctive RNA viruses can only

r plicate in the presence of helper virus. The helper virus and the defective virus need not be th sam retrovirus; for example, a leukemia virus can act as helper virus to a defective sarcoma virus.

Nondefective retroviruses contain a long terminal repeat (LTR) region at the 5' and 3' end of the genome, as well as gag, pol and env genes (reviewed in Watson et al., 1987, In Molecular Biology of the Gene, Vol. 2, Benjamin/Cummings Publishing Co., Menlo Park, CA and Hanafusa, 1977, In Comprehensive Virology, Fraenkel-Conrat, H. and Wagner, R.R., eds. Plenum Publishing Co., NY, pp. 401-436). However, with the exception of Rous sarcoma virus, isolates of nondefective retroviruses have not contained oncogenes. Nondefective retroviruses generally induce leukemias after fairly long latent periods (e.g., HTLV I, feline leukemia virus).

Natural isolates of defective retroviruses, virtually all of which contain oncogenes, result from deletions of gag, pol, and env genes (reviewed in Watson et al., supra; Hanafusa, supra). These viruses are known as acute transforming viruses and have the ability to induce tumors in vivo after a short latent period (e.g., sarcoma viruses). Furthermore, it has been shown that as long as the LTRs are intact, the proviral DNA can become integrated into the host genome, transcribed into RNA and consequently transform host cells, but cannot produce infectious progeny. However, if such cells are infected with nondefective viruses, a mixture of nondefective and defective viruses are produced. Often in this case pseudotypes are produced in which the envelope glycoprotein made by the non-defective virus is incorporated into the defective virus yielding infectious particles.

Defective retroviruses hav also be n used in the process of gene transduction in which the defective

virus carries foreign DNA sequencs. For example, the transduction of primary cultures of adult rat hepatocytes by replication-defective retroviruses that constitutively express high levels of β-galactosidase has been reported (Wilson et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:3014-3018).

2.2.3. DEFECTIVE DNA VIRUSES

DI particles have been observed to be generated from such DNA viruses as papovaviruses, herpes viruses, adenoviruses, and parvoviruses through aberrant viral replication events or homologous recombination (reviewed in Holland, supra). As with RNA viruses, these DI particles of DNA viruses are generated after high titer infection with wild-type virus. As with RNA viruses, DI particles of many different structures are also generated from DNA viruses which contain deletions, substitutions, and duplications of the wild-type genome.

DI particles have also been observed to attenuate acute virus infection in papovaviruses (Brochman, 1977, Proc. Med. Virol. 23:69-85) and adenoviruses (Larsen, 1982, Virology, 116:573-580). However, like RNA viruses, DI particles from DNA viruses are thought to be involved in persistent infection. For example, DI genomes replicate autonomously in cells transformed by BK virus, a human papovavirus (Yogo et al., 1980, Virology 103:241-244).

2.3. VACCINES

2.3.1. INACTIVATED OR ATTENUATED PATHOGENS

Traditional methods of preparing vaccines include the use of inactivated or attenuated viruses. Inactivation of the virus renders it harmless as a biological agent but does not destroy its imunogenicity. Injection of these "killed" virus

particles into a host will then elicit an immune response capable of neutralizing a futur inf ction with a live virus. However, a major concern in the use of killed vaccines (using inactivated virus) is failure to inactivate all the virus particles. Even when this is accomplished, since killed viruses do not multiply in their host, the immunity achieved is often short lived and additional immunizations are usually required. Finally, the inactivation process may alter the viral proteins rendering them less effective as immunogens.

Attenuation refers to the production of virus strains which have essentially lost their disease producing ability. One way to accomplish this is to subject the virus to unusual growth conditions and/or frequent passage in cell culture. Viral mutants are then selected which have lost virulence but yet are capable of eliciting an immune response. The attenuated viruses generally make good immunogens as they actually replicate in the host cell and elicit long-lasting immunity. However, insufficient attenuation can be problematic.

2.3.2. SUBUNIT VACCINES

An alternative to the above methods is the use of subunit vaccines (for example, see U.S. Patent No. 3,636,191 by Blumberg and Millman). This involves immunization only with those proteins which contain the relevant immunological material. One advantage of subunit vaccines is that the irrelevant viral material is excluded. For many enveloped viruses, the virally encoded glycoprotein contains those epitopes which are capable of eliciting neutralizing antibodies. The HBV subunit vaccine contains the hepatitis B surface antigen (HBsAg) purified from the blood of chronically infected carriers (Krugman, 1982, J.A.M.A. 247:2012-2015). This vaccine has been shown to be

effective and safe in high risk adult populations, e.g., drug addicts and homosexuals and in newborn infants. However, the vaccine is very expensive due to the finite supply of serum, complex purification and invactivation process as well as the lengthy safety test in chimpanzees required for its production and certification.

Subunit vaccines may also be prepared using synthetic peptides representing immunologically imported domains of surface antigens. This approach has been attempted with a number of viruses, e.g., foot and mouth disease (Bittle et al., 1982, Nature (London) 298:30-33, influenza A virus (Muller et al., 1982, Proc. Natl. Acad. Sci. U.S.A. 79:569-573), poliovirus (Emini et al., 1983, Nature (London) 304:699-703) and HBV (Itoh et al., 1986, Proc. Natl. Acad. Sci. 83:9174-9178). The peptide is coupled to a carrier (e.g., hemocyanin, Poly-DL-alanine) and administered in conjunction with an adjuvant. There are some disadvantages to this approach. These peptides generally do require the use of Freund's adjuvant, a carcinogen, to potentiate a strong immune response, since they are relatively poor antigens.

The use of recombinant DNA technology for the production of subunit vaccines involves the molecular cloning and expression in an appropriate vector of the viral genetic information coding for those proteins which can elicit a neutralizing response in the host animal.

A number of methods for producing viral surface antigen in eukaryotic cells have been developed for HBV and other viruses. One example involves the transfection of yeast with a recombinant in which HBsAg is inserted into a yeast expression vector downstream from an inducible pr moter (reviewed in Valenzuela et al., 1988, Patent No. 4,722,841). Yeast, however, must be disrupted to release HBsAg.

Th antigen is then purified by isopycnic and rate zonal centrifugation combined with immunoaffinity chromatography.

Analogous approaches have been attempted in mammalian cells using recombinant viruses. Generally, a virus is used as a vector to express foreign genes inserted into its genome. Upon introduction into host animals, the recombinant virus expresses the inserted foreign gene and may thereby elicit a host immune response to such gene product. HBsAg as well as other surface antigens have been produced using recombinant vaccinia virus (Smith et al., 1983, Nature (London) 302:490-495), an SV40 recombinant with a defective origin of replication (Burnette et al., 1984, In: Modern Approaches to Vaccines, Lerner, R.A. and Chanock, R.M. (eds.) Cold Spring Harbor, NY pp. 245-250), bovine papilloma virus (Statowa et al., supra, pp. 239-243), and adenoviruses (Morin et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:4626-4630). In addition, an SV40 recombinant has been constructed in which HBsAg replaces the region coding for the SV-40 VP1 protein (Levinson et al., 1988, U.S. Patent No. 4,741,901).

2.4. GENE THERAPY

Gene therapy refers to the transfer and stable insertion of new genetic information into cells for the therapeutic treatment of diseases or disorders. Usually, the foreign gene is transferred into a cell that proliferates to spread the new gene throughout the cell population. Thus stem cells, or pluripotent progenitor cells, are usually the target of gene transfer, since they are proliferative cells that produce various progeny lineages which will potentially express th foreign gene.

Most studies in gen therapy have focused on the use of hematopoietic stem cells. High effici ncy g ne

transfer systems for h matopoietic progenitor cell transformation have been investigated for use (Morrow, 1976, Ann. N.Y. Acad. Sci. 265:13; Salzar et al., 1981, In Organization and Expression of Globin Gene, A.R. Liss, Inc., New York, p. 313; Bernstein, 1985, In Genetic Engineering: Principles and Methods, Plenum Press, New York, p. 235; Dick et al., 1986, Trends in Genetics 2:165). Reports on the development of viral vector systems indicate a higher efficiency of transformation than DNA-mediated gene transfer procedures (e.g., calcium phosphate precipitation and DEAE dextran) and show the capability of integrating transferred genes stably in a wide variety of cell types. Recombinant retrovirus vectors have been widely used experimentally to transduce hematopoietic stem and progenitor cells. Genes that have been successfully expressed in mice after transfer by retrovirus vectors include human hypoxanthine phosphoribosyl transferase (Miller et al., 1984, Science 255:630) and human β-globin (Dzierzak, E.A., et al., 1988, Nature (London) 331:35-41). Bacterial genes have also been transferred into mammalian cells, in the form of bacterial drug resistance gene transfers in experimental models. The transformation of hematopoietic progenitor cells to drug resistance by eukaryotic virus vectors, has been accomplished with recombinant retrovirus-based vector systems (Hock and Miller, 1986, Nature (London) 320:275-277; Joyner et al., 1983, Nature (London) 305:556-558; Williams et al., 1984, Nature (London) 310:476-480; Dick et al., 1985, Cell 42:71-79; Keller et al., 1985, Nature (London) 318:149-154; Eglitis et al., 1985, Science 230:1395-1398). Adenoassociated virus vectors have been used successfully to transduce mammalian cell lines to neomycin resistance (Hermonat and Muzyczka, 1984, supra; Tratschin et al., 1985, Mol. Cell. Biol. 5:3251). Other viral vector systems that have been

investigated for use in gene transfer include papovaviruses and vaccina viruses (see Cline, 1985, Pharmac, Ther. 29:69-92).

Other methods of gene transfer include microinjection, electroporation, liposomes, chromosome transfer, and transfection techniques (Cline, 1985, supra). Salser et al., used a calcium phosphate precipitation transfection technique to transfer a methotrexate-resistant dihydrofolate reductase (DHFR) or the herpes simplex virus thymidine kinase gene, and a human globin gene into murine hematopoietic stem cells. In vivo expression of the DHFR and thymidine kinase genes in stem cell progeny was demonstrated (Salser et al., 1981, In Organization and Expression of Globin Genes, Alan R. Liss, Inc., New York, pp. 313-334).

Gene therapy has also been investigated in murine models with the goal of enzyme replacement therapy. Thus, normal stem cells from a donor mouse have been used to reconstitute the hematopoietic cell system of mice lacking beta-glucuronidase (Yatziv et al., 1982, J. Lab. Clin. Med. 90:792-797). Since a native gene was being supplied, no recombinant stem cells (or gene transfer techniques) were necessary.

3. SUMMARY OF THE INVENTION

The present invention is directed to replication defective hepadnaviruses. In particular, the invention relates to two types of defective hepadnavirus genomic DNA, and the nucleic acid sequences thereof. The first type (termed herein "particle-defective" hepadnavirus genomes) are incapable of supplying all hepadnaviral functions required for replication, but are able to produce a pregenomic RNA with the appropriate cis-acting signals required for inclusion of the RNA in virions (packaging) and for reverse transcription into DNA.

In a specific embodiment, hepadnaviruses containing such a particle-defective genome can be defective in production of core antigen (HBcAg), viral polymerase, and/or surface antigen (HBsAg). The second type of defective hepadnavirus genomes according to the present invention (termed herein "packaging genomes") are genomes which can be transcribed into pregenomic RNAs that are incapable (i) of being reverse-transcribed into a double-stranded genomic DNA, and/or (ii) of being packaged themselves, yet which genomes can be transcribed into mRNAs that encode protein(s) which are able to supply an in trans viral function necessary for packaging a second RNA into virions or for transcribing the second RNA into virion DNA. In a specific embodiment of the invention, such packaging genomes include but are not limited to genomic DNA which contains a deletion in the direct repeat (DR) region, DR1.

Hepadnavirus virion particles containing a particle-defective genome can be produced by coexpression of the particle-defective genome and "helper" hepadnavirus packaging genome(s). Such virion can also be produced by coexpression of the particle-defective genome and nucleic acid vector(s) (e.g., plasmids) which encode hepadnaviral in trans packaging functions (e.g., reverse transcriptase, core, envelope functions). The resulting hepadnavirus, containing a particle-defective genome ("packaged particle-defective genome") can then be used for the infection of a hepatocyte, to which the particle-defective DNA is delivered and in which it can be expressed, but which viral DNA cannot then bring about another round of hepadnavirus infection due to its defective nature. Thus, the particle-defective hepadnavirus genom can be expressed by infected hepatocytes in the absence of

further hepatitis virus multiplication and resultant diseases or disorders.

The present invention also relates to therapeutic uses for both packaged particle-defective genomes and packaging genome products. Both packaged particle-defective genomes and hepadnavirus packaging genome products which express immunogenic epitopes (e.g., HBsAg), may be formulated as vaccines for the prevention of hepadnavirus infection or used as immunotherapeutic agents. In one embodiment, such viruses may be used as immunostimulatory agents for the treatment of hepadnavirus infection or its sequelae.

The present invention is also directed to packaged recombinant particle-defective genomes which comprise a heterologous gene sequence, and the therapeutic uses of such viruses. In one embodiment of the invention, various regions of the hepadnavirus genome which include but are not limited to those sequencs encoding the viral functions that can be complemented in <u>trans</u> by the packaging genomes can be replaced with a heterologous gene sequence which will be expressed under the control of viral or other regulatory sequences when introduced into a given organism. Such recombinant viruses may be used for genetic therapy of enzyme deficiencies which can be treated by hepatic enzyme production (e.g. blood coagulation factors). In another embodiment of the invention, recombinant hepadnaviruses containing a heterologous gene may be formulated as vaccines for protection against infection by a pathogenic organism or for protection against conditions or disorders caused by the presence of an antigen.

In a further embodiment of the invention, packaged particle-defective g nomes can be used as agents which interfere with propagation or maintenance

of infection by wild-typ virus, and/or eradicating or mitigating wild-type virus infection.

Further embodiments of the invention relate to permanent hepatic cell lines stably transfected with defective hepadnaviruses. Cell lines which stably incorporate one or more defective hepadnaviral genomes are capable of supplying necessary functions to defective hepadnaviral genomes and producing defective infectious particles. In a specific embodiment, permanent cell lines are stably transfected with a hepadnavirus packaging genome, whereby such cell lines become capable of permanently expressing products required in trans for packaging and reverse transcription of a second defective genome (termed herein a "packaging cell line"). Introduction of a particle-defective genome into a packaging cell line permits recovery of progeny packaged particle-defective genomes.

3.1 DEFINITIONS

As used herein, the following terms shall have the meanings indicated:

bp	=	base pairs
DHBV	=	duck hepatitis B virus
DR	=	direct repeat region, e.g., DR1 or DR2, of a hepadnavirus genome
FBS	=	fetal bovine serum
GSHBV	=	ground squirrel hepatitis B virus
HBV	=	hepatitis B virus (humans)
HBcAg	=	core antigen of DHBV, GSHBV, HBV or WHBV
HBsAg	=	surface antigen of DHBV, GSHBV, HBV or WHBV
kb	=	kilobases
kd	=	kilodaltons
NTP	=	nucleoside triphosphate
WHBV	=	wo dchuck hepatitis B virus

DR1 = a DHBV packaging genome, having a deletion of the 12 bp direct repeat termed DR1 which is required for complete synthesis of a double-stranded viral DNA molecule

particle-defective hepadnavirus genome = a defective hepadnavirus genomic DNA that does not encode all <u>in trans</u> packaging functions, yet can be transcribed into a pregenomic RNA with the appropriate cis-acting signals required for inclusion of the RNA in virions (packaging) and for reverse transcription into DNA

hepadnavirus packaging genome = a defective hepadnavirus genomic DNA which is transcribed into pregenomic RNA that is incapable of being (a) packaged, and/or (b) reverse-transcribed into a double-stranded DNA genome, yet whose encoded messenger RNAs encode one or more proteins which can supply in <u>trans</u> viral functions necessary for packaging an appropriate second pregenomic RNA into virions and for transcribing the second RNA into virion DNA.

Packaging cell line = a permanent cell line stably incorporating at least one hepadnavirus packaging genome, which cell line is capable of expressing viral products necessary for <u>in trans</u> packaging of an appropriate

second pregenomic RNA into virions and reverse transcribing the second RNA into viral DNA.

Producing cell line = a permanent cell line containing the genetic information necessary for substantially continuous production of infectious hepadnaviral particles containing a defective hepadnaviral genome.

4. DESCRIPTION OF THE FIGURES

Figure 1. Diagrammatic representation of the structural and functional features of DHBV DNA as well as of major transcripts (a); structure of particle-defective DHBV genomes (b); structure of the DR1 packaging genome (c); and the structure of λ-DHBV recombinant genomes (d).

Figure 2. Immunofluorescent staining of HuH7 cells transfected with particle-defective DHBV DNA. Cells were transfected with the following DNAs: RV^-718 (pol^-), RV^-2650 ($core^-$), Kpn^- (pol^-env^-), or Kpn^-Sph^- ($pol^-env^-core^-$), and stained after five days incubation for core (α-c) or surface (α-S) antigens. Magnification: 40X.

Figure 3. Southern blot hybridization assays of the appearance of viral replicative DNA in HuH7 cells transfected with DNA of pol^-env^-, and $pol^-env^-core^-$ viral mutants and λ-DHBV recombinants. Plates (60 mm) of HuH7 cells (2-3 X 10^6 cells/dish) were transfected with DNA of wild-type DHBV (lanes 1,3), wild-type + Kpn^-Sph^- ($pol^-env^-core^-$) (lanes 2,4), DR1 (lane 5), ΔDR1 + Kpn^-+1000 (lane 7), ΔDR1 + Kpn^-+180 (lane 8), ΔDR1 + Kpn^-Sph^- ($pol^-env^-core^-$) (lane 9). Southern hybridization analysis was carried out as described in

Section 6.4.2, infra, with a DHBV RNA probe of positive polarity. Lane 10 contained 1 picogram cloned DHBV DNA as a hybridization standard. Lane 11 contains λ-HindIII molecular size markers. Exposure times were 4 hours (lanes 1 and 2) or 48 hours (lanes 3-11). The positions of relaxed circular (RC) and single-stranded (SS) forms of intracellular viral DNA are shown at left.

Figure 4. Immunofluorescent staining of HuH7 cells transfected with the packaging genome, Δ DR1. Cells transfected with wild-type DHBV DNA (WT) or Δ DR1 DNA (ΔDR1) were stained for core (α-C) or surface (α -S) antigens. PC: Phase contrast photograph. Magnification: 40X.

Figure 5. Viral replicative forms in HuH7 cells after transfection with packaging genomes and particle-defective DHBV genomes. Viral DNA was assayed as described for figure 3. Cells were transfected with DNA of wild-type DHBV (lane 1), Δ DR1 (lane 2), Δ DR1 + Kpn$^-$ (pol$^-$env$^-$) (lane 3), Δ DR1 + RV$^-$718 (pol$^-$) (lane 4), Δ DR1 + RV$^-$ 2650 (core$^-$) (lane 5), RV$^-$2650 (core$^-$) (lane 6), RV$^-$2650 (core$^-$) + Kpn$^-$ (pol$^-$ env$^-$) (lane 7), RV$^-$718 (pol$^-$) (lane 8), RV$^-$2650 (core$^-$) + RV$^-$718 (pol$^-$) (lane 9). The positions of relaxed circular (RC) and single-stranded (SS) forms of intracellular viral DNA are shown at left.

Figure 6. Assay of transfected HuH7 cell culture supernatants for infectious virus. Supernatant fluids (2 ml) from HuH7 cells transfected with 1.5, 3 or 6 μg wild-type DHBV DNA (lanes 1, 2, 3, respectively) or 3 μg of DNA of Δ DR1 (lanes 4,5), Kpn$^-$ (pol$^-$env$^-$) (lanes 6,7), or Kpn$^-$Sph$^-$ (pol$^-$env$^-$core$^-$) (lanes 8,9) were incubated overnight with cultures of primary duck hepatocytes. Infected hepatocytes were incubated for 12 days and total DNA was extracted and assayed for viral replicative DNA forms by Southern blot hybridization as describ d for figure 3. Lane 10

contained λ-HindIII mol cular size markers. The positions of relaxed circular (RC) and single-stranded (SS) DNA are shown at left.

Figure 7. Suppression of infectious virus production by DHBV mutant DNAs in cotransfection of HuH7 cells. Supernatant fluids from HuH7 cells transfected with viral DNAs were assayed for infectivity by Southern hybridization as described for figure 6. Viral DNA was obtained from hepatocytes infected by incubation with culture fluids of cell transfected with DNA of wild-type DHBV (lanes 1,2), wild-type + Kpn⁻ (pol⁻env⁻) (lanes 3,4), wild-type + Kpn⁻Sph⁻ (pol⁻env⁻core⁻) (lanes 5,6), wild-type + ΔDR1 (lanes 7,8), or wild-type + pSP65 (lanes 9,10).

Figure 8.(a) Nucleotide sequence of wild-type DHBV genome between positions 1326 and 1350, and the corresponding sequence of the particle-defective genome 1S, described in Section 6.7, infra.

(b) Viral replicative forms. In lanes 1 and 2, Southern blot hybridization assay of HuH7 cells following transfection with a dimerized form of the particle-defective genome 1S. Supernatants from the HuH7 cells transfected with either dimerized mutant 1S genome (lane 5) or 1S dimer plus ΔDR1 dimer (lane 7) were used to infect primary duck hepatocytes and the cells were then assayed for viral DNA. Southern blot hybridization assay was performed as described for Figure 3. Lanes 3, 4, 6, and 8 represent controls, respectively: Lane 3 -- duck hepatocytes infected with serum from duck with active DHBV hepatitis; Lane 4 -- duck hepatocytes infected with fluids from WT transfection of HuH7 cells; Lane 6 -- duck hepatocytes infected with supernatant fluid from WT + ΔDR1 cotransfection of HuH7 cells; and Lane 8 -- duck hepatocytes, nontransfected.

Figure 9. Immunofluorescent staining of primary duck hepatocytes following infection with wild-type DHBV (column 1), and with supernatant fluids recovered from HuH7 cells cotransfected with the defective genomes ΔDR1 and 1S (column 2), and stained for presence of core antigen. Magnification: 40X

Figure 10. The plasmid pHBVΔDR1 Neo, showing the orientation and relative positions of the dimerized ΔDR1 sequence, the selectable marker, and certain restriction sites. R = EcoRI, B=BamHI, SV=SV40 regulatory DNA.

Figure 11. Flow chart representation of the construction steps for preparation of the plasmid pHBVΔ DR1Neo. Amp^R=ampicillin resistance gene; CAM^R=chloramphenicol resistance gene; R=EcoRI; restriction sites are as illustrated.

Figure 12. Transmission electron microscopy of thin section of Hep B1-2 cells. Magnification: 125,000X.

Figure 13. Representation of the deduced arrangement of ΔDR1 DNA sequences, derived from the plasmid pHBVΔDR1Neo, as integrated into host cellular DNA in HepB1-2 cells. R=EcoRI; B=BamHI; pBR=pBR322 sequences; serrated lines=cellular sequences.

Figure 14. Southern blot hybridization analysis of nucleic acids isolated from different density gradient fractions of supernatant from Hep B1-2 cell cultures using a nick-translated HBV probe. Supernatant fractions were separated by centrifugation in cesium chloride, and nucleic acids isolated from the fractions, as described in Section 8.2, infra. Gradient range values are in grams/milliliter.

Figure 15. Southern blot hybridization analysis of nucleic acids isolated from supernatants of Hep B1-2 cells transfected with the particle-defective genome designated X-. Transfections were performed with 5 micrograms (lanes

1-3) and 1 microgram (lanes 4-6) of X- plasmid DNA. Supernatants were fractionated, and nucleic acids isolated from fractions, as described in Section 8.2, infra.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to replication-defective hepadnaviruses. In particular, the invention relates to two types of defective hepadnavirus genomes and the nucleic acid sequences thereof. The first type (termed herein "particle-defective" genome) is incapable by itself of supplying all the hepadnaviral functions required for replication, but is able to produce a pregenomic RNA with the appropriate cis-acting signals required for inclusion of the RNA in virions (packaging) and for reverse transcription into DNA. In specific embodiments, hepadnaviruses containing such a particle-defective genome can be defective in the synthesis of core antigen (HBcAg), viral polymerase, and/or surface antigen (HBsAg). The second type of defective hepadnavirus genome according to the present invention (termed herein "packaging genome") is a genome which can be transcribed into a pregenomic RNA that is incapable of being reverse-transcribed into a double-stranded DNA genome and/or being packaged itself, yet which genome can be transcribed into messenger RNA(s) which encode one or more proteins that are able to supply in trans viral functions necessary for packaging a second pregenomic RNA into virions or for transcribing the second RNA into virion DNA. Thus, packaging genomes can provide "helper function," to package particle-defective genomes. Packaging genomes include but are not limited to those hepadnavirus genomes which contain deletions in the direct repeat (DR) regions, DR1 and/or DR2.

The particle-defective hepadnavirus genomes of the invention, which in a specific embodiment contain heterologous gene sequences, can be packaged by expression of hepadnavirus proteins required in trans for packaging (e.g. core, reverse transcriptas , env lope) which the particle-defective genome itself

cannot supply in functional form. Such expression results in the production of a hepadnavirus virion particle containing a particle-defective genome (termed herein "packaged particle-defective genome" or "particle-defective virus"). In one embodiment, such in trans packaging function(s) can be provided by expression of genes of one or more nucleic acid vectors (e.g. plasmids) which encode protein(s) supplying such function(s). In another embodiment, the particle-defective genomes can be packaged by the expression of one or more hepadnavirus packaging genomes. Thus, in one particular embodiment, when packaging genomes and particle-defective genomes are introduced together into permissive cells in vitro by gene transfer techniques, expression of the packaging genomes supplies the functions needed to permit the particle-defective genome to be included in viral particles that are subsequently released from the cells. In an alternative specific embodiment, packaging of particle-defective genomes can be accomplished by the introduction of the particle-defective genomes into a cell line in which the "helper" packaging genome(s) are already stably incorporated and expressed.

The particle-defective DNA-containing virions ("particle-defective viruses") that are produced can then be used for the infection of a hepatocyte or other susceptible cell, in vitro or in vivo, to which cell the particle-defective DNA is delivered and in which it can be expressed, but which viral DNA cannot then bring about another round of hepadnavirus infection due to its defective nature. Thus, the particle-defective hepadnavirus genome can be expressed by infected hepatocytes in the absence of further hepatitis multiplication and resultant diseas s or disorders.

In a specific mbodiment, a limited round of hepadnavirus multiplication may be induced in a host cell incorporating a particle-defective genome, by the administration of a conditional replication-defective hepadnavirus (e.g., a helper virus genome under the control of an inducible promoter), which would act as a "helper" virus only under certain conditions. Such a limited viral multiplication could provide for further, yet limited and non-pathogenic spread, of the particle-defective virus DNA to other host cells. In an embodiment of the invention in which a recombinant particle-defective viral DNA is used for purposes of genetic therapy, such a conditional viral propagation may be preferred.

In a specific embodiment of the invention, defective hepadnaviruses can be used as antiviral agents in the treatment of hepadnavirus infection. Such viruses can be formulated as interfering agents that inhibit propagation or maintenance of wild-type virus.

In another embodiment, packaged particle-defective viruses or hepadnavirus packaging genome products, which express immunogenic epitopes (e.g., HBsAg), may be formulated as vaccines for the prevention of hepadnavirus infection or used as immunostimulatory agents.

The present invention is also directed to particle-defective recombinant hepadnavirus genomes which comprise a heterologous gene sequence, viruses containing such genomes, and the therapeutic uses of such viruses. Various regions of the hepadnavirus genome which include but are not limited to those encoding the viral functions that can be complemented in trans by the packaging genomes can be replaced with a heterologous gene s quence, such that the heterologous sequ nce will be expressed under the control of hepadnaviral or heterologous regulatory

sequences when introduced into the appropriat cell. In an embodiment in which the heterologous DNA sequence encodes an enzyme whose deficiency is the basis of disease or disorder, viruses containing these recombinant genomes may be used for genetic therapy of such deficiencies which can be treated by hepatic enzyme production. In another embodiment of the invention, recombinant hepadnaviruses containing a heterologous gene sequence encoding an immunogenic epitope may be formulated as vaccines for protection against infection by the heterologous organism or for protection against conditions or disorders caused by the presence of an antigen.

5.1 GENERATION OF AND USES FOR DEFECTIVE HEPADNAVIRUS GENOMES

The present invention is directed to replication-defective hepadnavirus genomes that include but are not limited to particle-defective genomes which may be packaged by functions supplied in trans, and packaging genomes, which are capable of supplying packaging functions in trans. The invention is also directed to nucleic acid sequences encoding the sequence of the particle-defective or packaging genomes.

Packaged particle-defective hepadnavirus genomes include but are not limited to hepadnavirus mutants defective in the synthesis of core antigen (HBcAg), viral polymerase, and/or surface antigen (HBsAg). In a specific embodiment, the defect in expression may be due to a deletion encompassing a portion of or the entire coding region for the given protein(s). In specific embodiments of the invention, the packaged particle-defective genomes are defective in the production of ne, two, or all three of the above hepadnavirus proteins.

The hepadnavirus packaging genomes of the invention include but are not limited to those genomes with mutations or deletions in regions required in cis for viral DNA synthesis, or those required in cis for recognition and packaging of the pregenomic RNA. In one embodiment, such packaging genomes can have deletions in the DR1 (direct repeat 1) or DR2 region. In another specific embodiment, the hepadnavirus genome has a deletion in the DR1 region. In another embodiment of the invention, the packaging genome can have deletions in both the DR1 and DR2 regions. The DR1 and DR2 regions have been shown to be involved in the initiation of viral DNA synthesis.

5.1.1. GENERATION OF DEFECTIVE HEPADNAVIRUS GENOMES

Defective hepadnavirus genomes of the present invention can be generated through the use of recombinant DNA techniques. The types of defective hepadnaviruses whose DNA can be used include but are not limited to hepatitis B virus (HBV), pathogenic in humans and higher primates, ground squirrel hepatitis B virus (GSHBV), duck hepatitis B virus (DHBV), and woodchuck hepatitis B virus (WHBV). The generation of defective hepatitis genomic DNA according to the present invention may be divided into the following steps, solely for the purpose of description. The first step involves the isolation of wild-type hepatitis virus DNA. The hepadnavirus DNA may be obtained from hepadnavirus infected hepatocytes or from cloned DNA by known techinques (see, e.g., Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). In a second step, the isolated hepadnavirus DNA is treated to generate a mutation that will render the virus replication-defective. Aft r religation of the viral genome, if nec ssary, and infection or transformation

of susceptible cells, the defective viruses may be identified by: (a) nucleic acid hybridization (b) presence or absence of "marker" gene functions, or (c) expression of defective hepadnavirus genes.

The wild-type hepadnavirus genomic DNA may be obtained from hepadnaviruses such as HBV, DHBV, GSHBV, and WHBV. These genomic DNAs have been cloned and sequenced (Seeger et al., 1984, J. Virol. 51:367-375; Mandart et al., 1984, J. Virol. 49:782-792; Galibert et al., 1982, 41:51-65; and Valenzuela et al., 1980, In: Animal Virus Genetics, Field, B.N., Jaenisch, R. and Fox, C. F., eds., Academic Press, NY, pp. 57-70). If the cloned hepadnavirus DNA is not readily available, hepadnavirus DNA may be obtained from hepatocytes infected with hepadnavirus (See, e.g., Tuttleman et al., 1986, J. Virol. 58:17-25). Alternatively, HBV, GSHBV, DHBV or WHBV DNA may be obtained from purified virus using standard procedures known in the art (See, e.g., Mason et al., 1980, J. Virol. 36:829-836). The hepadnavirus DNA may subsequently be cloned by standard procedures known in the art (see, e.g., Maniatis, T., et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Nucleotide sequence analysis of the cloned gene can be carried out by various procedures known in the art, e.g., the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, F., et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, CA).

Many strategies known in the art can be used in the construction of both particle-defective genomes and hepadnavirus packaging genomes. In the generation f particle-defective genomes, the viral DNA is treated enzymatically or chemically to generate a mutant virus that is replication-defective, yet which

retains the signals required in *cis* for packaging. In a preferred embodiment, the viral DNA is treated with one or more restriction enzymes to cleave the DNA at specific sites and then religated. If an appropriate deletion resulting in cohesive DNA termini are generated by restriction endonuclease digestion, no further modification of DNA before ligation may be needed. If, however, cohesive termini of the DNA are not available for generation by restriction enzyme digestion, or if deletion of additional sequence is desired, enzymatic cleavage can be followed by digestion with a nuclease such as nuclease Bal 31, exonuclease III, λ exonuclease, mung bean nuclease, or T4 DNA polymerase exonuclease activity, to name but a few, in order to remove portions of the DNA sequence. Enzymatically treated DNA termini can be modified to facilitate ligation of the viral DNA by any of numerous techniques known in the art. For example, cleavage with a restriction enzyme can be followed by modification to create blunt ends by digesting back or filling in single-stranded DNA termini before ligation. An oligonucleotide sequence (linker) which encodes one or more restriction sites can be ligated to the cleaved ends of viral DNA. The hepadnavirus sequences are then ligated *in vitro*, either to each other, or to appropriate expression vector sequences.

In a preferred embodiment, the generation of particle-defective hepadnaviruses involves the deletion of a gene or genes coding for the surface antigen, core antigen, and/or polymerase/surface antigen. Part of the region encoding the polymerase protein also codes for the surface antigen. More than one restriction enzyme may be used in the generation of deletion(s). In one embodiment of the invention, hepadnavirus DNA coding for two of the viral proteins are removed by restriction enzyme digestion. In another embodiment of the invention, DNA encoding all

three proteins are removed by restriction enzyme digestion. Alternatively, the restriction enzyme digestion(s) may only remove a portion of the hepadnavirus DNA coding for HBsAg, HBcAg, or the polymerase protein, with the resulting mutant expressing a dysfunctional product, or being deficient in the expression of the protein encoded by the attenuated gene. Again, deletions may be generated in one, two, or three of the above-mentioned hepadnavirus genes.

In an alternative method of the invention, particle-defective hepadnavirus genomes may be generated by mutation in vitro or in vivo. In one embodiment, such particle-defective genomes may be deficient in the expression of a viral protein due to transcriptional (e.g., promoter) or translational defects. In another embodiment, a hepadnavirus genome may be rendered particle-defective due to a mutation (e.g., point or frameshift) in a viral protein which prevents the protein's proper functioning. For example, a core protein that is unable to assemble may be produced. Any technique for mutagenesis known in the art can be used, including but not limited to in vitro site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

The generation of hepadnavirus packaging genomes can be accomplished by mutation in the DR1 region (see Section 6.3, infra). It is also envisioned that such mutants can be generated by deletion in the DR2 region. Such techniques as discussed supra for the generation of particle-defective genomes can be used in the generation of hepadnavirus packaging genomes. In a preferred embodiment, the DR1 region, or a portion thereof, can be deleted by restriction enzyme digestion. Confirmation that the appropriate genetic mutation has been achieved may be accomplished by

techniques known in the art, e.g., by restriction mapping and/or sequence analysis.

The particle-defective genomes and packaging genes can, if desired, be inserted into various nucleic acid vectors. In a preferred embodiment of the invention, the particle-defective genomes or hepadnavirus packaging genomes are inserted into a plasmid cloning vector which is used to transform appropriate host cells in order to replicate the DNA so that many copies of the hepadnavirus sequences of interest are generated. This can be accomplished by ligating the hepadnavirus sequence into a cloning vector which has complementary cohesive termini. If, however, cohesive termini of the DNA are not available for generation by restriction endonuclease digestion, or different sites other than those available are preferred, any of numerous techniques known in the art may be used to accomplish ligation of the defective hepatitis virus DNA at the desired sites. For example, as mentioned supra, cleavage with a restriction enzyme can be followed by modification to create blunt ends by digesting back or filling in single-stranded DNA termini before ligation. Alternatively, the cleaved ends of the defective hepatitis DNA can be "chewed back" using a nuclease in order to remove portions of the sequence. An oligonucleotide sequence (a linker) which encodes one or more restriction sites can be inserted in a region of the hepadnavirus DNA by ligation to DNA termini. A linker may also be used to generate suitable restriction sites in hepadnavirus sequences. Additionally, hepatitis virus sequences can be mutated in vitro or in vivo in order to form new restriction endonuclease sites or destroy preexisting ones, to facilitate in vitro ligation procedures.

In a particular embodiment of the invention, the genomes of the defective hepatitis viruses may be

dimerized using standard procedures known in th art (see Maniatis, T., et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). For example, a large excess of the defective hepadnavirus DNA in relation to the vector DNA may be used in the ligation reaction.

Transformation of host cells with recombinant DNA molecules that incorporate the hepadnaviral DNA sequence enables generation of multiple copies of the hepadnaviral DNA. Thus, the hepadnaviral DNA may be obtained in large quantitites by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

Once the necessary sequences for the generation of hepadnavirus particle-defective or packaging genomes have been isolated, they may be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, retrovirus, SV40, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA. The expression elements of these vectors vary in their strength and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, (e.g., mouse metallothionien promoter) or from viruses that grow in these cells, (e.g., vaccinia virus 75 kb promoter) may

be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Specific initiation signals are also required for efficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the protein coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic.

Methods used for the insertion of defective hepadnavirus DNA into vectors may include in vitro recombinant DNA and synthetic techniques for such expression vectors as plasmids or bacteriophages or in vivo recombinations (genetic recombination) for virus expression vectors such as vaccinia virus or adenovirus.

If the ultimate goal is to insert the hepadnaviral sequences into virus expression vectors such as vaccinia virus, adenovirus, or retrovirus, the recombinant DNA molecule that incorporates the particle-defective hepadnavirus or hepadnavirus packaging genome can be modified so that the gene is flanked by virus sequences that allow for genetic recombination in cells infected with the virus expression vector, so that the hepadnaviral DNA can be inserted into the other viral genome.

Vectors containing sequences of particle-defective hepadnavirus genomes or of hepadnavirus packaging genomes can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expr ssion of inserted sequences. In the first approach, th presence of a modified hepadnavirus genome inserted in an expression

vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the hepadnavirus sequence. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus etc.) caused by the insertion of foreign genes in the vector. For example, if the modified hepatitis DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the modified hepadnavirus genome can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of the gene product. For example, an ELISA can be used to detect the presence of antigenic determinants reactive with antibodies to HBsAg, HBcAg, or the hepadnaviral polymerase protein.

Once the hepadnavirus sequences have been identified, the expression vector comprising the hepadnavirus sequences should then be transferred into an appropriate host. This can be accomplished by any of numerous methods known in the art including but not limited to transformation (e.g., when the vector is a plasmid), phase transduction, calcium phosphate mediated transfection (e.g., mammalian cell virus vectors) or microinjections. A host cell may be chosen which modulates the expression of the inserted sequences, or modifies and proceses the chimeric gene product in the specific fashion desired. Expression from certain promoters can b elevated in the presence of certain inducers (e.g., zinc and cadmium ions for methallothi nein promoters). Furthermor ,

modifications (.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of th encoded prot in(s). Different host cells have characteristic and specific mechanisms for the posttranslational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed.

5.1.2. PACKAGING OF PARTICLE-DEFECTIVE GENOMES

The particle-defective hepadnavirus genome may be packaged into hepadnavirus virion particles (termed herein "packaged particle-defective genomes"). This packaging may be accomplished by supplying hepadnavirus gene products required in trans for packaging. Thus, nucleic acid vectors (expression vectors) which express such hepadnaviral gene products can be used to provide in trans packaging function(s). In another embodiment, the particle-defective genome can be packaged by the use of the encoded products of a packaging genome. Such packaging genome products can be supplied, e.g., by cotransfection of packaging genomes with particle-defective genomes into susceptible cells (e.g., hepatocytes, leukocytes) or by the delivery of particle-defective genomes (e.g., by transfection) into a packaging cell line in which the packaging genome has been stably incorporated and is expressed. Virion particles representing packaged particle-defective genomes may then be isolated. The virion particles may be purified using procedures known in the art (for example, see Tuttleman et al., 1986, J. Virol. 58:17-25). Alternatively, the hepadnavirus DNA may be used, e.g., therapeutically, while still cloned into a given vector.

5.1.3. USES FOR DEFECTIVE HEPADNAVIRUS GENOMES AND PACKAGED PARTICLE-DEFECTIVE GENOMES

Particle-defective hepadnavirus genomes, hepadnavirus packaging genomes, and their gene products may be used in the prevention and/or treatment of diseases or disorders due to hepadnaviruses. In one embodiment of the invention, vaccines can be formulated from packaging particle-defective genomes (packaged, e.g., by packaging genome expression), or from hepadnaviral virion particles devoid of nucleic acid (resulting from packaging genome expression), which express immunogenic epitopes of hepatitis virus. The types of viruses which can be used include but are not limited to HBV, DHBV, GHBV, and WHBV.

In another embodiment of the invention, defective hepadnavirus virion particles containing a heterologous gene sequence may be used in vaccine formulations for protection against heterologous pathogens, or in gene therapy approaches.

In yet another embodiment of the invention, packaged particle-defective genomes may be used as therapeutic agents in the treatment of acute or chronic manifestations of hepatitis resulting from hepadnavirus infection. In this embodiment of the invention, these defective viruses act by interfering with the replication of wild-type hepadnavirus (see Section 6.7, infra).

5.1.3.1. HEPADNAVIRUS VACCINE FOR THE PREVENTION OF INFECTION

Vaccines for the prevention of hepadnavirus infection may be formulated according to the present invention from defective hepadnaviruses which express an immunogenic epitope, for example, one or more of the epitopes of core antigen and/or surface antigen, which provides protective immunity upon administration to the host. In a preferred embodiment, an epitope of the surface antigen is expressed.

In one embodiment of the invention, a vaccine can be formulated from th protein products of hepadnavirus packaging genome expression. For example, "empty" virion particles (i.e., those lacking genomic DNA due to the packaging genome's inability to package its own pregenomic RNA and/or to synthesize its own genomic DNA) can be used in vaccine formulations. As one example, such empty virions can be harvested from the supernatants of cells transfected with DNA of a packaging genome.

In another embodiment of the invention, the vaccine can be formulated from packaged particle-defective genomes. In this embodiment, an immune response would be generated against epitopes produced by expression of the packaged genome upon introduction into a susceptible cell. Once introduced into such a cell, the particle-defective genome will be expressed, yet will not initiate another round of infection due to its defective nature. In one embodiment, the vaccine can be formulated from a packaged particle-defective genome whose DNA has deletions in the gene encoding the core antigen. In another embodiment, the particle-defective genome can have deletions in the 5' region of the gene encoding the viral polymerase protein, thereby inhibiting the expression of this protein, but not the surface antigen. The hepadnavirus vaccine may be formulated in yet another embodiment of the invention from packaged particle-defective DNA with deletions in both the region encoding the core antigen and the 5' end of the polymerase gene.

5.1.3.1.1. DETERMINATON OF IMMUNOPOTENCY OF THE EPITOPE(S) EXPRESSED ON DEFECTIVE HEPADNAVIRUSES

The immunopotency of the epitope expressed by a virion comprising a packaged particle-defectiv

genome, or by an "empty" (i.e., lacking genomic nucleic acid) virion (produced by hepadnavirus packaging genome expression) in a vaccine formulation can be determined by monitoring the immune response of test animals following immunization with the packaged particle-defective genome or hepadnavirus packaging genome particle. Test animals may include chimpanzees and other primates and eventually human subjects (for HBV immunization). Methods of introduction of the immunogen may include oral, intradermal, interamuscular, intraperitoneal, intravenous, subcutaneous, intranasal or any other standard routes of immunization.

The immune response of the test subjects can be analyzed by various approaches such as: (a) the reactivity of the resultant immune serum to hepadnaviral antigen or a fragment thereof containing the hepadnavirus epitope, or to the isloated naturally occurring hepadnavirus, as assayed by known techniques, e.g., enzyme-linked immunosorbant assay (ELISA), immunoblots, radioimmunoprecipitations, etc., (b) the reactivity of lymphocytes isolated from the immunized subject to the hepadnaviral antigen or fragment thereof, or the virus as assayed by known techniques, e.g., blastogenic response assays, cytotoxicity assays, delayed type hypersensitivity, etc., (c) the ability of the immune serum to neutralize infectivity of hepadnavirus in vitro and (d) protection from disease and/or mitigation of infectious symptoms in immunized animals.

5.1.3.1.2. FORMULATION OF A VACCINE

The purpose of this embodiment of the invention is to formulate a vaccine in whch the immunogen comprises an epitope of a given hepadnavirus so as to elicit an immune (humoral and/or cell mediated) response to the hepadnavirus epit pe that will protect

against infection by a given hepadnavirus or against diseases or disorders caused by an hepadnavirus. Such a vaccine may be univalent or multivalent.

Multivalent vaccines can be preared from a singel or a few hepadnavirus virion particles which express one or more hepadnavirus epitopes. Alternatively, multivalent vaccines can be formulated to contain hepadnavirus gene products and/or recombinant viruses when the defective hepadnavirus genome is cloned into a mammalian or insect cell virus vector.

Two main types of immunogens are envisioned for use in the vaccine formulations of the present invention: (i) hepadnavirus virion particles, or (ii) isolated hepadnavirus proteins (for use in subunit vaccines). In one embodiment, the hepadnavirus virion particles can comprise packaged particle-defective genomes, resulting in a virion particle that can undergo only a single round of infection on its own. In this embodiment, the immunogenic epitopes are produced by expression of the packaged hepadnavirus genome upon introduction into a susceptible cell. In another embodiment, the hepadnavirus virion particle used as immunogen can comprise an "empty shell" devoid of viral DNA (produced by the introduction into a cell and expression therein of an hepadnavirus packaging genome). Unlike the packaged particle-defective genomes, such an empty virion will not cause the synthesis of hepadnaviral proteins upon introduction into a host cell.

Many methods may be used to introduce the vaccine formulations of the invention; these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transcutaneous, and intranasal routes, as well as the natural route of infection of the parent wild-type hepadnavirus.

5.1.3.1.3. USE OF ANTIBODIES DIRECTED AGAINST DEFECTIVE HEPADNAVIRUSES

The antibodies generated against hepatitus virus by immunization wth defective hepatitis virus particles or proteins thereof of the present invention also have potential uses in diagnostic immunoassays, passive immunotherapy, and generation of antiidiotypic antibodies.

The generated antibodies may be isolated by standard techniques known in the art (e.g., immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays. The antibodies may also be used to monitor treatment and/or disease progression. Any immunoassay system known in the art, such as those listed supra, may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, protein A immunoassays, fluorescent immunoelectrophoresis assays, to name but a few.

The vaccine formulations of the present invention can also be used to produce antibodies for use in passive immunotherapy, in which short-term protection of a host is achieved by the administration of pre-formed antibody directed against a heterologous organism.

The antibodies generated by the vaccine formulations of the present invention can also be used in the production of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunizati n, in order to produce a subpopulation of antibodies that bind the initial antigen of the

pathogenic microorganism (Jerne, 1974, Ann. Immunol. (Paris) 125c:373; Jerne, et al., 1982, EMBO J. 1:234).

5.1.3.2. USES AS IMMUNOTHERAPEUTIC AGENTS

In this embodiment of the invention, an effective amount of hepadnavirus virion particles of the invention (as described in Section 5.1.2.1.2), or proteins encoded by the defective hepadnavirus genomes, which comprise an immunogenic epitope, may be used immunotherapeutically. In one embodiment, the immunogenic epitope can be of the core antigen. In a preferred embodiment, the epitope can be of the surface antigen.

Hepadnavirus virion particles or hepadnavirus proteins which contain an immunogenic epitope may be therapeutically effective in the prevention of hepatoma, by virtue of their ability to stimulate an immune response against hepadnavirus. Experimental evidence indicates a correlation between hepadnavirus infection and the incidence of hepatoma on both an epidemiological and molecular level. The immunogens may be administered alone or concurrently with other therapies (e.g., chemotherapy, radiation therapy, etc.).

In a particular embodiment of this invention, defective hepadnavirus virion particles or proteins containing an immunogenic epitope may be used as immunostimulators to boost the host's immune system, enhancing cell mediated immunity, and facilitating the clearance of a given infectious agent (e.g., hepatitis A virus, cytomegalovirus). In a preferred embodiment, since hepadnaviruses are generally found in large concentrations in hepatocytes, blood, and bone marrow of infected individuals, packaged particle-defective genomes may be administered alone or in conjunction with other therapies in the treatment of diseases that affect the above types of cells (e.g., thos caused by

hepatitis A virus, malaria parasites, cytomegalovirus, Epstein Barr Virus, etc.)

5.1.3.3. TREATMENT FOR HEPADNAVIRUS INFECTION

Packaged particle-defective hepadnavirus genomes may be used as antiviral agents in the treatment of acute or chronic hepadnavirus infection due to their ability to interfere with the replication of wild-type hepadnaviruses. One embodiment of the invention involves hepadnaviruses that produce defective surface antigen, core antigen, or viral polymerase protein. In a preferred embodiment of the invention, the particle-defective hepadnavirus is deficient in the synthesis of viral polymerase protein, and encodes both a truncated surface antigen protein and a truncated core antigen protein. The particle-defective hepadnavirus may be administered alone or concurrently with other antiviral agents which include but are not limited to α-interferon, and vidarabine phosphate.

5.2. GENERATION OF AND USES FOR PACKAGED RECOMINANT PARTICLE-DEFECTIVE GENOMES COMPRISING A HETEROLOGOUS SEQUENCE

The present invention also relates to packaged particle-defective genomes comprising a heterologous gene sequence. In one embodiment of the invention, various regions of the hepadnavirus genome which include but are not limited to those encoding the viral functions that can be complemented in trans by the packaging genomes, can be replaced with a heterologous cloned gene in such a way that the heterologous gene will be expressed under the control of viral or other regulatory sequences when introduced into a given organism. In one embodiment of the invention, heterologous DNA sequences are inserted into the surface antigen-encoding region of a

h padnavirus. In a further embodiment of the invention, heterologous DNA sequences are inserted into the core antigen region of a hepadnavirus. The heterologous sequences can be expressed under the control of hepadnaviral promoters. In an alternative embodiment, heterologous transcriptional regulatory regions can be used, by construction of the appropriate recombinant DNA.

These recombinant hepadnaviruses have a number of therapeutic applications. In one embodiment of the invention, these recombinant hepadnaviruses may be used for gene therapy in the treatment of diseases and disorders affecting the liver, or of disorders which may be treated by enzyme production in the liver (see Section 5.2.2.1, infra). For example, such diseases may result from an inherited deficiency of a hepatic enzyme(s) or a blood coagulation factor, or infection by a pathogenic organism. In another embodiment of the invention, the heterologous gene may code for a hepatic enzyme(s) or a product which is toxic to a given pathogen that is the causative agent of a disorder.

5.2.1. GENERATION OF RECOMBINANT HEPADNAVIRUS GENOMES CONTAINING HETEROLOGOUS SEQUENCES

Recombinant hepadnavirus genomes can be generated through the use of recombinant DNA techniques known in the art. The hepadnavirus genomes which can be used include but are not limited to hepatitis B virus (HBV), which infects humans and higher primates, ground squirrel hepatitis B virus (GSHBV), duck hepatitis B virus (DHBV), and woodchuck hepatitis B virus (WHBV).

The generation of recombinant hepadnavirus genomes may be divided into the following three steps solely for the purpose of description: 1) isolation of the heterologous sequence, 2) construction of

recombinant hepadnavirus DNA, and 3) expression of recombinant hepadnavirus genomes.

5.2.1.1. ISOLATION OF THE HETEROLOGOUS SEQUENCE

Any heterologous DNA sequence which encodes a protein product whose expression in hepatocytes is desired can be isolated for use. In one embodiment of the invention directed to the treatment of enzyme deficiencies (see Section 5.2.2.1, infra) any heterologous DNA sequence which encodes a functional enzyme whose deficiency is the basis of a disorder can be isolated for use (for non-limiting examples of such enzymes, see Table II infra in Section 5.2.2.1). In another specific embodiment of the invention, the DNA sequence may encode a blood coagulation factor for use in gene therapy of inherited disorders of coagulation, since the liver is the normal site of the biosynthesis of such factors. The isolated DNA sequence may encode the whole protein sequence of a given enzyme or a functional portion of the sequence representing the active site.

The heterologous DNA sequence may encode a gene product that ameliorates disease. In one embodiment, directed to the treatment of liver disorders resulting from infection by a pathogenic organism, the heterologous gene sequence may encode a product that is toxic to a pathogen without significant detriment to the host, or which interferes with a pathogen's life cycle, etc. in another embodiment of the invention. Such DNA sequences may encode an altered gene product of a given pathogen or an "anti-sense" sequence (i.e., one that can hybridize to functional nucleic acid of the pathogen).

A further embodiment of the invention directed to vaccine use (see S ction 5.2.2.2 infra) involves the isolation of a DNA sequence which encodes an epitope of a h terologous organism, which when introduced into

an appropriate host, produces protective immunity against such an organism or against a condition or disorder caused by an antigen of the organism. In this embodiment of the invention, packaged recombinant particle-defective genomes comprising a heterologous gene sequence may be formulated as vaccines to prevent diseases caused by a pathogenic organism containing the heterologous gene sequence. Such pathogenic organisms include but are not limited to those listed in Table I.

TABLE I

PATHOGENIC ORGANISMS WHOSE EPITOPE(S) MAY BE ENCODED BY RECOMBINANT HEPADNAVIRUSES

Epstein Barr virus
Hepatitis A virus
Non-A, non-B hepatitis virus
Cytomegalovirus
Plasmodium spp. (malaria parasites)

In a preferred embodiment, the heterologous sequence of the recombinant hepadnavirus encodes an epitope of a pathogenic microorganism that is hepatotropic.

In a specific embodiment, any DNA sequence which encodes an epitope of a malaria parasite of the genus Plasmodium which is immunogenic in a vertebrate host can be isolated for use according to the present invention. The species of Plasmodium which can serve as DNA sources include but are not limited to the human malaria parasites P. falciparum, P. malariae, P. ovale, P. vivax, and the animal malaria parasites P. berghei, P. yoelii, P. knowlesi, and P. cynomolgi. The antigens or fragments th reof which can be expressed by recombinant hepadnaviruses are epitopes which are expressed by the malaria parasite at any of

the various stages in its lif cycle. In a particular embodiment, the heterologous epitope to be expressed is an epitope of the circumsporozoite (CS) protein of a species of Plasmodium. (See, e.g., Dame et al., 1984, Science 225:593; Arnot, et al., 1985, Science 230:815; Weber et al., 1987, Exp. Parasitol. 63:295).

Other epitopes which can be expressed by recombinant hepadnaviruses include but are not limited to the following: epitopes on the hepatitis A antigen (von der Helm et al., 1981, J. Virol. Methods 3:37-43); neutralizing epitopes on the 64-66 envelope glycoprotein of CMV (Plotkin, 1985, In The Hepresviruses, Roizman, B. and Lopez, C. Plenum Press, N.Y., pp. 297-312); and neutralizing epitopes on the EBV membrane antigen protein, gp 340 (North et al., 1982, Proc. Natl. Acad. Sci. U.S.A. 79:7504-8508).

The gene sequences encoding the heterologous epitope to be expressed according to the present invention, can be isolated by techniques known in the art including but not limited to purification from genomic DNA of the microorganism, by cDNA synthesis from RNA of the microorganism, by recombinant DNA methods (Maniatis, T., et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), or by chemical synthesis.

5.2.1.2. CONSTRUCTION OF RECOMINANT HEPADNAVIRUS GENOMES

The construction of a recombinant hepadnavirus genome according to this embodiment of the invention involves the replacement of sequences in the hepadnavirus genome with a sequence encoding one or more epitopes of a heterologous organism. In one embodiment of the invention, the heterologous gene sequence replaces the genes coding for the surface antigen and viral polymerase protein. In another

embodiment of the invention, the heterologous gene sequence replaces the gene coding for the surface antigen. The recombinant hepadnavirus DNA in this embodiment can retain pre-S DNA sequences which contain promoters for surface antigen expression. In yet another embodiment of the invention, the heterologous gene replaces the gene coding for the core antigen. The recombinant hepadnavirus DNA of this embodiment can also contain pre-C sequences that promote core antigen expression.

Many strategies known in the art can be used in the construction of the recombinant hepadnavirus gene. For example, the relevant sequences of the hepadnavirus genome and of the heterologous DNA can, by techniques known in the art, be cleaved at appropriate sites with restriction endonuclease(s), isolated, and ligated in vitro. If cohesive termini are generated by restriction edonuclease digestion, no further modifdication of DNA before ligation may be needed. If, however, cohesive termini of the DNA are not available for generation by restriction endonuclease digestion, or different sites other than those available are preferred, any of numerous techniques known in the art may be used to accomplish ligation of the heterologous DNA at the desired sites as discussed supra (see Ssection 5.1.1).

The particular strategy for constructing gene fusions will depend on the specifc hepadnavirus sequence to be replaced or inserted into, as well as the heterologous sequence to be inserted.

5.2.1.3. EXPRESSION OF THE RECOMBINANT HEPADNAVIRUS GENOMES

Hepadnavirus genomes containng heterologous gene inserts can be identifi d by three general approaches: (a) nucleic acid hybridization, (b) presence or abs nce of "mark r" gene functions, and (C) xpression

of ins rted sequences. In th first approach, the presence of a foreign gene inserted in an hepadnavirus vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the foreign inserted gene. In the second approach, the recombinant vector/host system can be identifed and selected based upon the presence or absence of certain "marker" gene functions caused by the insertion of foreign genes in the hepadnavirus vector. For example, if the heterologous gene is inserted within the polymerase gene sequence of the hepadnavirus, recombinants containing the heterologous insert can be identified by the absence of polymerase activity. In the third approach, recombinant hepadnaviruses can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of the gene product.

In the particular embodiment of the invention detailed in the examples infra, the construction, expression, and packaging of a recombinant hepatitis genome contaiing a heterologous gene sequence that replaces hepatitis viral polymerase and surface antigen genes is described. This packaged recombinant particle-defective genome requires for its propagation the concurrent introduction of a "helper" hepatitis virus packaging genome into a given cell line. Such packaging genomes, an example of whose construction is detailed in Section 6.3, are capable of providing in trans viral functions necessary for packaging an appropriate pregenomic RNA into virions and transcribing the RNA into virion DNA.

Once a hepadnavirus recombinant which expresses a heterologous gene is identified, the gene product should be analyzed. This can be achieved by assays based on the physical, immunological or functional

properties of the product known in the art. The peptide or prot in encoded by the heterologous DNA may be isolated and purifed by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

5.2.2. USES FOR RECOMBINANT HEPADNAVIRUSES CONTAINING A HETEROLOGOUS GENE SEQUENCE

5.2.2.1. GENE THERAPY

Recombinant particle-defective genomes and their recombinant hepadnaviruses (i.e., packaged recombinant particle-defective genomes) which contain a heterologous gene sequence can be therapeutically valuable. The stable incorporation by host cells (e.g., hepatocytes) of a recombinant hepadnavirus genome containing a heterologous gene sequence capable of expression by the host cells, can be of great value in the treatment of diseases and disorders.

Numerous techniques are known in the art and may be used for the introduction of the defective recombinant hepadnavirus DNA into host cells (e.g., hepatocytes), for the purposes of gene therapy. The technique used should provide for the stable trnasfer of the hepadnavirus sequence to host cells, which may include but are not limited to hepatocytes, lymphocytes, and leukocytes, so that the recombinant hepadnavirus sequence is expressible by the above mentioned cells, and so that the necessary developmental and physiological functions of the recipient cells are not disrupted. In a preferred embodiment, recombinant particle-defective hepadnavirus DNA is packaged into virions (e.g., through the use of helper packaging genome expression); such a virion particl can th n provide

for stabl transfer of its contained recombinant DNA to a host cell through the noraml route of hepadnavirus infection. Routes of administration of virion particles which may be used include but are not limited to intrahepatic, oral, intravenous, subcutaneous, intraperitoneal, intramuscular, intradermal, intranasal, etc.

In specific embodiments of the invention, recombinant hepadnaviruses may be used to treat disorders of metabolic hepatic dysfunction. In one embodiment, these recombinant hepadnaviruses may be used in the treatment of liver disorders which are due to inherited deficiencies of hepatic enzymes. Such genetic disorders include but are not limited to those listed in Table II (for a detailed discussion of some of these disorders, see Sharp, 1985, In Gastroenterology, Berk, J.E., ed., W.B. Saunders Co., Philadelphia, pp. 3236-3258).

TABLE II

DISEASES OR DISORDERS WHICH CAN BE TREATED BY EXPRESSION OF PARTICLE-DEFECTIVE GENOMES CONTAINING A HETEROLOGOUS GENE SEQUENCE

I. Inherited Deficiencies of Liver Enzymes
   A. Hyperammonemia
      1. Inherited urea cycle enzyme defects
         a. N-acetyl glutamate (AGA) synthetase deficiency
         b. Carbamyl phosphate synthetase (CPS I) deficiency
         c. Ornithine carbamyl transferase (OCT) deficiency
         d. Citrullinemia
         e. Arginosuccinic aciduria
      2. Inherited organic acidemias from catabolism of branched chain amino acids,

odd chain fatty acids, and th side chain of cholesterol

a. Propionic acidemia
b. Methylmalonic acidemia
c. Isovaleric acidemia

B. Infantile cholestasis
1. Galactosemia
2. α-antitrypsin deficiency
3. Trihydroxycoprostanic acidemia

C. Hematomegaly (enlarged liver)
1. Glycogen storage disease - 4 types classified according to enzyme deficiency
2. Fructose intolerance
3. Tyrosinemia
4. Acid lipase deficiency

D. Liver disease after early childhood
1. Hemochromatosis
2. Wilson's disease

II. Inherited Disorders of Coagulation
A. Hemophilia A - Factor VIII deficiency
B. Hemophilia B - Factor IX deficiency
C. Rare hereditary disorders of coagulation (e.g. fibrinogen defects, factor V defects, factor XI defects, etc.)

Other proteins which can be encoded by recombinant hepadnavirus genomes include but are not limited to complement components, medium chain acyl CoA dehydrogenase, low density lipoprotein receptor, insulin, digestive enzymes, etc.

In another embodiment of the invention, expression of recombinant hepadnavirus genomes encoding a blood coagulation factor may be used to treat inherited coagulation disorders including but not limited to those listed in Table II. For a detailed description of thes disorders, see Jandl,

J.H., 1987, In Blood, Textbook of Hematology, Little Br wn and Co., Boston, MA, pp. 1095-1140. Current therapies involve replacement therapy of a given factor isolated from the plasma of individuals possessing normal levels of the factor. However, the patient receiving the replacement risks transmission by the donor of blood-borne viral infections. The use of this embodiment of the invention would eliminate such a risk. The liver is the site of biosynthesis of factors involved in coagulation such as fibrinogen, factors VIII, IX, X, XI, XII, and XIII, and plasminogen. In a specific embodiment, one or more of these factors can be supplied by expression of a recombinant hepadnavirus particle-defective genome.

In a specific embodiment of the invention directed to the treatment of alcoholic liver disease, a recombinant hepadnavirus can be used which expresses a mutant alcohol dehydrogenase that has a lower K_m for alcohol, thus accelerating the production of acetaldehyde, the agent responsible for making the alcohol consumer very ill, thus inhibiting drinking behavior.

Another embodiment of the invention involves treating patients with liver disorders resulting from infections by pathogenic microorganisms, with recombinant hepadnaviruses. Such recombinant hepadnaviruses can contain a heterologous gene which is expressed as a product which ameliorates disease, is toxic to the pathogen without significant detriment to the host, or interferes with the pathogen's life cycle, etc. Pathogens which cause disorders which may be treated with recombinant hepadnaviruses according to this embodiment of the invention include but are not limited to those pathogens listed in Table I, supra. The heterologous gene may code for an altered gene product of the pathogenic organism.

Alternativly, it is possible to construct a recombinant hepadnavirus that expresses a sequ nce which is "anti-sense" to the nucleic acid of a hepatocyte pathogen. Such a sequence, which is complementary to the pathogen's RNA or DNA, can hybridize to and inactivate such RNA or DNA, inhibiting the function or expression of the nucleic acid and disrupting the pathogen's life cycle.

Many other heterologous sequences may be expressed by the recombinant particle-defective DNA of the invention for the purposes of gene therapy. For example, where a disorder results from the excess of a specific product, an inhibitor or degradative enzyme for the product, or an inhibitor of its snythesis, may be expressed. An antisense oligonucleotide can be expressed by recombinant hepadnavirus DNA for the purpose of blocking specific gene transcription.

5.2.2.2. VACCINE USE

According to this embodiment of the invention, recombinant hepadnavirus virions or proteins which comprise an immunogenic epitope of a heterologous organism are formulated for vaccine use. Such vaccines can be used to provide protection against infection by a heterologous pathogenic organism, including but not limited to those listed in Table I, supra, or for protection against conditions or disorders caused by an antigen of the organism. Such vaccine formulations can comprise live particle-defective hepadnavirus vaccines or subunit vaccine formulations. The hepadnavirus recombinants may be packaged by use of an approriate hepadnavirus packaging genome or other source of hepadnavirus protein, and the resulting virion formulated for use as a vaccine, or the expressed protein containng the heterologous epitop may be purified for use in a

subunit vaccine. Th vaccine formulations of the invention can be of use in animals and/or humans.

5.2.2.2.1. DETERMINATION OF IMMUNOPOTENCY OF THE HETEROLOGOUS EPITOPE(S) EXPRESSED BY RECOMBINANT HEPADNAVIRUSES

Immunopotency of the heterologous epitope expressed by a recombinant hepadnavirus, in its live vaccine formulation, can be determined by monitoring the immune response of test animals following immunization with recombinant hepadnaviruses. In a subunit vaccine formulation, the immune response of test animals can be monitored following immunization with the isolated heterologous product of the recombinant hepadnavirus, which can be formulated with an appropriate adjuvant to enhance the immunological response. Suitable adjuvants include, but are not limited to, mineral gels, e.g., aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum. Test animals may include chimpanzees and other primates, and eventually human subjects if the hepadnavirus is HBV. Other hepadnaviruses such as DHBV, GSHBV, and WHBV which have been constructed to contain an epitope of a heterologous organism may also be used in vaccine formulations to immunize ducks, ground squirrels or woodchucks respectively, against infection by the heterologous organism. Methods of introduction of the immunogen may include oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, or any other standard routes of immunization.

The immune response of the test subjects can be analyzed by various approaches such as: (a) the reactivity of the resultant immune serum to the native

antigen or a fragment thereof containing the heterologous epitope, or to the isolated naturally occurring heterologous organism, as assayed by known techniques, e.g., enzyme-linked immunosorbant assay (ELISA), immunoblots, radioimmunoprecipitations, etc., (b) the reactivity of lymphocytes isolated from the immunized subject to the native antigen or fragment thereof, or to the heterologous organism, as assayed by known techniques, e.g., blastogenic response assays, cytotoxicity assays, delayed type hypersensitivity, etc., (c) the ability of the immune serum to neutralize infectivity of the organism in vitro or the biologic activity of the native antigen, and (d) protection from disease and/or mitigation of infectious symptoms in immunized animals.

5.2.2.2.2. FORMULATION OF A VACCINE

In one embodiment of the invention, the recombinant hepadnaviruses which express an epitope of a heterologous organism are formulated for live vaccine use. In another embodiment, the protein products of such a recombinant virus can be formulated for use in subunit vaccines. The live vaccine or subunit vaccine may be univalent or multivalent. The vaccine formulations of the invention are of use in humans and/or animals.

5.2.2.2.2.1. LIVE VACCINE FORMULATIONS

The purpose of this embodiment of the invention is to formulate a vaccine in which the immunogen is a packaged recombinant particle-defective genome, comprising a sequence encoding an epitope of a heterologous organism which can be expressed in vivo so as to elicit an immune (humoral and/or cell mediated) response to the heterologous epitope that will protect against inf ction by the organism or against conditions or disorders caused by an antigen

of the organism. Such a live vaccine will allow introduction and expression of the particle-defectiv genome within a susceptible cell, but no subsequent round of infection will occur.

The live vaccine formulation can be univalent or multivalent. Multivalent vaccines can be prepared from a single or a few packaged recombinant particle-defective genomes which encode one or more heterologous epitopes, which may be of different organisms. A single recombinant hepadnavirus genome may encode more than one epitope of the same or different antigens.

Many methods may be used to introduce the live vaccine formulations of the invention; these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transcutaneous, and intranasal routes, as well as the natural route of infection of the parent wild-type hepadnavirus.

5.2.2.2.2.2. SUBUNIT VACCINE FORMULATION

As an alternative to live vaccines, the heterologous peptide or protein may be used as an immunogen in subunit vaccine formulations, which may be multivalent. As previously explained, subunit vaccines comprise solely the relevant immunogenic material necessary to immunize a host. Accordingly, these heterologous proteins, which may be recombinant fusion proteins, may be purified from recombinant hepadnaviruses that express the heterologous epitopes using procedures established in the art.

The purified protein(s) should be adjusted to an appropriate concentration, formulated with any suitable vaccine adjuvant and packaged for use. Suitable adjuvants include, but are not limit d to those described supra in Section 5.2.2.2.1. The immunogen may also be incorporated into liposomes, or

conjugated to polysaccharides and/or oth r polymers for use in a vaccine formulation.

Many methods may be used to introduce the vaccine formulations described above; these include, but are not limited to, oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous and intranasal routes.

5.3. USES OF ANTIBODIES DIRECTED AGAINST RECOMBINANT HEPADNAVIRUSES

The antibodies generated against heterologous organisms by immunization with the recombinant hepadnaviruses of the present invention also have potential uses in diagnostic immunoassays, passive immunotherapy, and generation of antiidiotypic antibodies.

The generated antibodies may be isolated by standard techniques known in the art (e.g., immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays to detect the presence of viruses, bacteria, or parasites of medical or veterinary importance in human or animal tissues, blood, serum, etc. The antibodies may also be used to monitor treatment and/or disease progression. Any immunoassay system known in the art may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immuno lectrophoresis assays, to name but a few.

The vaccine formulati ns of the present invention can also be used to produce antibodies for use in

passive immunotherapy, in which short-t rm protection of a host is achiev d by the administration of pre-formed antibody direct d against a heterologous organism.

The antibodies generated by the vaccine formulations of the present invention can also be used in the production of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunization, in order to produce a subpopulation of antibodies that bind the initial antigen of the pathogenic microorganism (Jerne, 1974, Ann. Immunol. (Paris) 125c:373; Jerne et al., 1982, EMBO J. 1:234).

6. GENERATION OF DEFECTIVE HEPADNAVIRUSES

In the examples detailed herein, the invention is illustrated by way of a duck model system in which two types of defective duck hapatitis B virus genomes are constructed. The first type, particle-defective DHBV genomes, contain deletions in the genes encoding the polymerase protein, core antigen, and/or surface antige (see figure 1). These genomes are incapable of replication by themselves, but are able in each case to produce a pregenomic RNA with appropriate cis-acting signals required for inclusion of the RNA in virions and for reverse transcription into DNA. The second type of defective DHBV genomes, packaging genomes, contain deletions in the DR1 region of the DHBV genome. These mutant genomes are capable of providing in trans all viral functions necessary for packaging an appropriate pregenomic RNA into virions and for transcribing the RNA into virion DNA. The mutant genomes however fail to be themselves reverse-transcribed into genomic DNA.

6.1 GENERAL PROCEDURES

6.1.1. CELLS

The human hepatoma cell line, HuH7 (Nakabayaski et al., 1982, Cancer Res. 42:3858-3863) was obtained from Dr. C. Koiki (Department of Gene Research, CancMer Institute, Tokyo, Japan) and was propagated in Dulbecco's Modified Eagle's Media (DMEM):F12 (GIBCO, Grand Island, NY) plus 10% fetal bovine serum (FBS).

Hepatocytes for establishing primary duck hepatocyte cultures were isolated from the livers of two week old Pekin ducks (obtained from Dr. W. Mason, Fox Chase Cancer Center, Philadelphia, PA) by collagenase perfusion (Tuttleman et al., 1986, J. Virol. 58:17-25). The primary hepatocytes were propagated in serum free L15 medium (GIBCO, Grand Island, NY) plus 10 mM sodium bicarbonate, 1.5% (v/v) dimethyl sulfoxide (DMSO), 5% FBS as well as supplements previously described (Tuttleman et al., supra). Cells were incubated at 37°C in an atmosphere containing 5% CO_2 (v/v).

6.1.2. PLASMIDS

The DHBV sequences used in the construction of defective DHBV genomes were obtained from DHBV genomic DNA cloned into a plasmid pSP65 vector at the EcoRI site, p5.2 Galxl, which was derived from a virion DNA clone used by Mandart et al. for the determination of the DHBV DNA nucleotide sequence (Mandart et al., 1984, J. Virol. 49:782-792).

6.1.3. PREPARATION, RESTRICTION, AND MODIFICATION OF DNA

Restriction enzynes, T4 DNA ligase, T4 DNA polymerase, and the Klenow fragment of E. coli DNA polymerase were purchased from New England Biolabs (Beverly, MA). Reactions with these enzymes w re carried out under conditions suggest d by the supplier unless otherwise specified.

After restriction enzyme digestion, DNA fragments were separated and purified by gel electrophoresis in a 5% polyacrylamide gel in TBE buffer (0.01 M Tris-borate, pH 8.2, 1 mM EDTA) or in 0.8% or 1.5% agarose gels in Tris-acetate buffer (0.02 M Tris-acetate, 0.05 M sodium acetate, 1 mm EDTA, pH 7.4). DNA fragments were visualized under ultraviolet light by negative shadowing over a PEI (polyethylene-imine) thin-layer chromatography plate or by ethidium bromide staining of gels. The DNA fragments were recovered by electroelution at room temperature for 2 hours at 200 V in TBE buffer, or were recovered from low-melt agarose gels by phenol extraction of the liquefied gel.

Oligonucleotides were snythesized on the 0.2 micromole scale, on an Applied Biosystems Inc. model 380B synthesizer, using beta-cyanoethyl phosphoramidite chemistry (Sinha et al., 1984, Nucl. Acids Res. 12:4539-4544). The oligonucleotides were separated and purified by gel electrophoresis using a polyacrylamide/8 M urea gel in the TBE buffer and subsequently visualized by negative shadowing over a PEI thin layer chromatography plate under ultraviolet light. The oligonucleotides were recovered by electroelution at room temperature for 2 hours at 200 V in TBE buffer.

6.2 CONSTRUCTION OF PARTICLE-DEFECTIVE DHBV GENOMES

The particle-defective DHBV genome containig deletions in regions encoding the polymerase and surface antigen (i.e., envelope) genes (pol^-, env^-) was generated by the digestion of the wild-type genome with KpnI, treatment with T4 polymerase in the presence of all four dNTPs, and closure of the plasmid with T4 DNA ligase (see figure 1b). The $pol^-env^-core^-$ mutant DNA (termed Kpn^-Sph^-) (figure 1b) with deletions in regions encoding all three viral genes was

produced by first digesting the DHBV genome with KpnI, resulting in the loss of the KpnI restriction site and generation of the Kpn⁻ genome. The Kpn⁻ genome was subsequently digested with SphI, then as before, treated with T4 polymerase in the presence of all four dNTPs. The plasmid was then closed with T4 DNA ligase.

The pol⁻ mutant genome (termed RV⁻718) or core⁻ mutant genome (termed RV⁻2650) (figure 1b) was generated by partial digestion of the wild-type genome with EcoRV. To accomplish this, 5 g of wild-type DHBV DNA was digested with 2 units of EcoRV for 15 minutes at 37°C. The partial digestion was followed by incubation in a T4 DNA polymerase reaction mixture in the presence only of dGTP.

All of the particle-defective DHBV genomes were dimerized using one of two strategies. The first employed partial EcoRI digestion in which 5 g DHBV DNA was digested with 2 units of EcoRI for 15 minutes at 37°C in order to linearize the plasmid at one of the two EcoRI sites flanking the genome. Linearized DNAs were identifed in 1% agarose gels by ethidium bromide fluorescense and recovered by electroelution in the TBE buffer at room temperature for 2 hours at 150 V. The isolated linearized plasmid was then treated with bacterial alkaline phosphatase and joined with the corresponding EcoRI-cut monomer genomes purified on a 5% nondenaturing polyacrylamide gel. The second stragegy of dimerization employed joining the EcoRI-cut gel-purified monomer in a 3 to 10-fold excess with EcoRI-bacterial aklaline phosphatase treated pSP65 DNA. Wild-type dimer plasmids can be prepared by either of these two methods from wild-type HBV or DHBV genomic DNA.

The defective DHBV genome cloned into pSP65 was amplified by transformation into E. coli HB101 cells.

The dimer clones were subsequently identified by restriction analysis of plasmid DNAs.

6.3 CONSTRUCTION OF DHBV PACKAGING GENOMES

A DHBV genome with a deletion of DR1, termed ΔDR1 genome (see figure 1c), was produced by first inserting the distal portion of the wild-type genome, contained between a BamHI site (at nucleotide number 1658) and an EcoRI site (at nucleotide number 3021), into a pBR322 derivative lacking an AccI restriction site. This was accomplished by AccI digestion, fill-in with Klenow enzyme, and ligation using T4 ligase, thus deleting the pBR322 sequence between nucleotide numbers 651 and 2246. Next, a portion of the wild-type DHBV genome which includes the DR1 region (nucleotide numbers 2535-2546) was excised from the recombinant plasmid using the enzymes AflII and AccI (cleaving at nucleotide numbers 2526 and 2577, respectively) and was replaced by a double-stranded synthetic DNA segment precisely lacking the 12 base pairs comprising DR1. Finally, the proximal portion of the DHBV genome (nucleotide numbers 0-1658), including adjoining plasmid sequences at the upstream end, was inserted as a BamHI fragment into the unique BamHI site.

The ΔDR1 genome, like the attenuated DHBV genomes, was dimerized using either of the two strategies outlined in Section 6.2. The ΔDR1 genome cloned into pSP65 was amplified by transformation into E. coli HB101 cells. The dimer clones were identified by the presence of a new HinfI site at the position of the DR1 deletion.

6.4 EXPRESSION OF DEFECTIVE DHBV GENOMES

Both particle-defective and packaging genome DHBV DNA (3μg) were transfected into HuH7 cells by the calcium precipitation procedure (Graham and van der

Eb, 1973, Virology 52:456-467). After five days, the expression of both particle-defective and packaging genome DHBV DNA was monitored for the presence of HBcAg and/or HBsAg. Cells were fixed at -20°C in 95% (v/v) ethanol:5% (v/v) glacial acetic acid. HBcAg and/or HBsAg was detected by indirect immunofluorescence using a rabbit antiserum to HBcAg or HBsAg (provided by Dr. W. Mason, Fox Chase Cancer Center, Philadelphia, PA) and rhodamine conjugated goat anti-rabbit IgG (Tuttleman, et al., 1986, J. Virol. 58:17-25).

6.4.1. EXPRESSION OF PARTICLE-DEFECTIVE DHBV GENOMES

The lack of production of core antigen by particle-defective DHBV DNA with deletions in regions encoding the core antigen (figure 2, RV$^-$2650) was confirmed by immunofluorescent staining (see figure 2). Immunofluorescent staining was also used to confirm the lack of production of surface antigen by particle-defective DHBV DNA with deletions in regions encoding the surface antigen and polymerase protein (Kpn$^-$ DNA) and in those with deletions in regions encoding the core antigen, surface antigen, and polymerase protein (Kpn$^-$Sph$^-$ DNA). The RV$^-$2650 mutant DNA was able to produce surface antigen, while the particle-defective DHBV with deletions in the region encoding the polymerase protein (RV$^-$718), and the Kpn$^-$ genome, were able to produce nucleocapsid (core) proteins as expected. Kpn$^-$Sph$^-$ encodes both a truncated surface antigen and a truncated core antigen, which were not detectable by immunofluorescence.

6.4.2. EXPRESSION OF PACKAGING GENOMES

As described supra, the packaging genome is deleted of a sp cific viral sequence, a 12 base pair direct repeat called DR1, which is required

for complete synthesis of a double-stranded viral DNA. The packaging genome was transfected into HuH7 cells by the calcium phosphate precipitation method (Graham and van der Eb, 1973, Virology 52:456-467). Five days after transfection, cells were washed with HBS (12 mM Hepes, 0.15 M NaCl, pH 7.45) and 1 ml of lysis buffer (10 mM Tris-hydrochloride (pH 7.9), 1 mM EDTA, 0.1% (w/v) NP-40, 50 mM NaCl, 8% (w/v) sucrose) was added directly to the cells. After incubation for 10 minutes at 37°C the lysate was removed and centrifuged at 13,000 rmp for 2 minutes in a Beckman Model B microfuge to pellet nuclei and other insoluble material. Viral core particles, which contain all the intermediates in DHBV DNA synthesis, where then isolated by the following procedure. The supernatant was removed and magnesium acetate ($MgAc_2$) was added to a final concentration of 6 mM. Deoxyribonuclease I (DNase I-Sigma Type II) was added at 100μl of 50 mM Tris-hydrochloride (pH 7.9), 6 mM $MgAc_2$ and 100μg/ml DNase I. After digestion of the suspension for 10 minutes at 37°C, viral cores were precipitated by the addition of 8% polyethylene glycol-8000 plus 0.5 M NaCl. The precipitate was collected by centrifugation at 4°C in a microfuge, and dissolved in 0.3 ml of buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 M NaCl, plus 100μg/ml DNase I. After 30 minutes at 37°C, the reaction mixture was adjusted to contain 15 mM EDTA, 0.5% SDS, and 500μg/ml pronase. After incubation at 37°C for 30 minutes, the sample was extracted with an equal volume of phenol. Nucleic acids were precipitated from the aqueous fraction with ethanol, dissolved in 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and subjected to electrophoresis in a 1.5% (w/v) agarose gel using conditions described by Tuttleman et al (1986, J. Virol. 58:17-25). Following electrophoresis, the gel was soaked for 30 minutes in 0.2 N NaOH, 1.5 M NaCl, then transferred to a nylon

membrane (Hybond N, Amersham) in 0.2 M Tris-hydrochloride (pH 4.0), 1.5 M NaCl, by the Southern blotting procedure (Southern, 1975, J. Mol. Biol. 98:503-517). After transfer, the filter was washed briefly in 2 x SSC then air dried and exposed to an ultraviolet light source (240-390 nm) for 5 minutes to covanently cross-link DNA to the membrane. The filter was hybridized at 50°C with a ^{32}p-labelled DHBV ribonucleotide probe of plus strand polarity. Autoradiography was performed at -80°C with Kodak X-OMAT AR film and an intensifying screen.

The results, shown in figure 3, lane 5, indicate that the complete relaxed circular DNA (RC) is not produced by this mutant DNA although some single stranded minus strand DNA (SS) is made. Replicative intermediates produced after transfection of the cells by the same amount of wild-type viral DNA are shown for comparison in lane 3.

Cells from these transfections were assayed for the production of viral envelope and nucleocapsid proteins by indirect immunofluorescent staining of the fixed cells (figure 4). ΔDR1-transfected cells appeared to produce amounts of the core (c) and surface (S) antigen equivalent to those produced by wild-type.

6.5 COMPLEMENTATION OF VIRAL FUNCTIONS REQUIRED FOR DNA SYNTHESIS BY ΔDR1

The ability of ΔDR1 to complement, in cotransfection experiments, known viral functions for viral DNA synthesis was tested. Simple frameshift mutant DNAs defective in core antigen (RV$^-$2650), in viral polymerase (RV$^-$718) and in both viral DNA polymerase and surface antigen synthesis (Kpn$^-$), as well as mutant DNAs deficient in the synthesis of all three known viral pr teins (Kpn$^-$Sph$^-$; see figure 1b) were cotransfected into HuH7 cells together with the

packaging genome, ΔDR1. In all cases, ΔDR1 was able to complement these defective genomes for the production of complete relaxed circular viral DNA (see figures 3 and 5).

In addition it was shown that these particle-defective DHBV genomes may also complement one another. For example, when the core⁻ mutant DNA was cotransfected with either the pol⁻ mutant or Kpn⁻ DNA, it was able to complement these DNAs for production of viral DNA (figure 5, lanes 7 and 9).

6.6 PRODUCTION OF INFECTIOUS VIRIONS BY TRANSFECTED HuH7 CELLS AND LACK OF INFECTIVITY OF SUPERNATANTS FROM DR1, Kpn⁻, AND Kpn⁻Sph⁻ TRANSFECTED CELLS

Cultured HuH7 human hepatoma cells can produce virions that are capable of infecting primary duck hepatocytes. A 100 mm dish was seeded with approximately 5 x 10^6 HuH7 cells and transfected after 24 hours with 1.5, 3, and 6 μg wild-type, cloned, dimeric DHBV DNA. Forty-eight hours after transfection, the medium (DMEM:F12 + 10% FBS) (GIBCO, Grand Island, NY) was replaced, and following incubation for a further 3 days, the culture medium was removed from the cells and distributed equally onto 3 x 60 mm dishes of primary duck hepatocytes prepared 48 hours earlier. The hepatocytes were infected for 20 hours at 37°C with the HuH7 culture medium. Cells were harvested as described by Tuttleman et al. (1986, J. Virol. 58:17-25) at 12 days post-infection, and total intracellular DNA was analyzed for the presence of DHBV DNA replication intermediates by Southern blot hybridization (see Section 6.4.2). Figure 6 shows the appearance of viral DNA in the cultures, indicating that infectious virus had been present in the HuH7 culture supernatants. Supernatants of cells transfected with Δ DR1 DNA alone, or with the Kpn⁻, or Kpn⁻Sph⁻ mutant

DNAs alone, failed to show the presence of replicative intermediates (figure 6). Therefore, HuH7 cells are capable of producing infectious DHBV from wild-type cloned DHBV DNA and, as expected, these cells failed to produce infectious virus from ΔDR1, Kpn⁻, or Kpn⁻Sph⁻ mutant DNAs because these mutants are replication-defective.

6.7 PRODUCTION OF INFECTIOUS VIRIONS FOLLOWING COTRANSFECTION OF CELLS WITH A ΔDRI GENOME AND A PARTICLE DEFECTIVE GENOME.

To examine for production of infectious virions from cells cotransfected with both a packaging genome and a particle-defective genome, a further particle-defective genome, designated S1, was constructed. Genome S1 was specifically designed to be defective for envelope production yet fully competent for DNA synthesis (e.g., env- pol+). Polymerase function was maintained in the S1 mutant to permit enhanced replication of viral DNA and enhanced production of viral gene products relative to a particle defective genome defective in both envelope and polymerase functions (e.g. Kpn-).

The S1 particle-defective genome was prepared from wild-type DHBV genomic DNA (Section 6.1.2, supra) by replacing the KpnI-XbaI fragment located between nucleotide positions 1290 and 1358 of the wild-type sequence with a synthetic linker of corresponding size. The synthetic linker contained specific nucleotide substitutions between positions 1326 and 1350, as shown in Figure 8(a). The particular mutations made to the wild-typ sequence to generat S1, shown in Figure 8(a), create termination codons in the nvelope open reading frame without changing the amino acid coding of the overlapping

polymerase open reading frame. A plasmid form of the 1S particl -d fective genome, d signated pSPDHBV.1S, was constructed by dimerzing the 1S genome within a pSP65 vector using methods described in Section 6.2.

The dimer-containing plasmid pSPDHBV.1S was transfected in HuH7 cells (Section 6.1.1., supra) by calcium precipitation. Transfected cells were assayed by Southern blot hybridization for viral replicative forms using the procedure described in Section 6.4.2., and the results are shown in Figure 8(b), lanes 1 and 2. As shown in Figure 8(b), lanes 1 and 2, HuH7 cells transfected with the 1S defective genome contain substantially wild-type levels of both the relaxed circular (RC) and single-stranded (SS) replicative forms of viral DNA. Immunostaining analysis of the transfected HuH7 cells (not shown) revealed accumulation of core antigen and an absence of detectable surface antigen.

Due to the absence of detectable levels of surface antigen in the 1S-transfected HuH7 cells, the transfectants are incapable of producing infectious viral particles. The absence of infectious particles in the supernatants of the 1S-transfected HuH7 cells was confirmed by recovering supernatants from the cultured transfectants and using them to infect cultures of primary duck hepatocytes (Section 6.1.1., supra). When the infected duck hepatocyte cultures were assayed by Southern blot hybridization for viral replicative forms, no viral DNA was detected. Figure 8(b), lane 5.

In order to assess the complementation of the 1S defective genome by the packaging genome ΔDR1, Δ DR1 plasmid dimer (Section 6.3., supra) was cotransfected into HuH7 cells together with the plasmid pSPDHBV.1S. After three days, th supernatant culture medium was recovered and used to infect cultures of primary duck hepatocytes. The infected

duck hepatocytes were harvested at twelve days post-inf ction and total intracellular DNA was assayed by Southern blot hybridization (Section 6.4.2.) for the presence of DHBV replicative intermediates. As seen in Figure 8(b), lane 7, new viral DNA synthesis could be detected in the infected duck hepatocytes as both relaxed circular and single-stranded forms of viral DNA. Immunostaining of the infected duck hepatocyte cell layers for the presence of core antigen (Figure 9) revealed levels of detectable core antigen equal to or greater than levels observed in control cells infected with wild-type DHBV. Immunostaining of the same cell population with anti-envelope antisera failed to reveal any detectable surface antigen, consistent with the env^- genotype of the 1S genome. The results indicate that the ΔDR1 genome can supply in trans the envelope function lacked by the particle-defective 1S genome, and that HuH7 cells are capable of producing infectious particles following cotansfection with both a particle-defective genome and a packaging genome. The resulting infectious particles are capable of infecting primary duck hepatocytes to produce viral replicative forms within the duck hepatocytes.

6.8 INTERFERENCE WITH WILD-TYPE VIRUS PRODUCTION

In order for a recombinant DHBV genome to serve as a vector for transducing heterologous DNAs, DHBV sequences necessary for transcription, packaging, and reverse transcription of the vector pre-genome must be present in the defective transducing genome. Since the mutant genomes pol^-env^- (Kpn^-), $pol^-env^-core^-$ (Kpn^-Sph^-), pol^-, and $core^-$ can be complemented in trans for DNA synthesis by ΔDR1, they must contain these necessary sequences. However, some of these mutations, which lie in viral open reading frames, appear to interfere in a dominant fashion with the synthesis of DNA and inf ctious particles by wild-type

virus. An example of such interference is shown in figure 3 (lanes 1,2 or 3,4). Wh n the Kpn^-Sph^- mutant DNA is cotransfected into HuH7 cells with wild-type DNA using procedures described in section 6.6, viral DNA synthesis is suppressed approximately 20-fold. We also observed that core antigen production, as detected by immunofluorescent staining is also suppressed. When cultrre fluids from such cotransfections were analyzed for infectivity of primary duck hepatocytes, we observed a strong reduction in the titer of infectious virus released from Kpn^-Sph^- + wild-type cotransfected cells compared to wild-type transfected cells (figure 7, lanes 1 and 2 compared with lanes 5 and 6). A similar but weaker effect could be seen with Kpn^- mutant genome (figure 7, lanes 1 and 2 compared with lanes 3 and 4). The intereference of some mutant DNAs with wild-type virus replication may offer an explanation for why we observe different efficiencies with which various mutants can be complemented.

7. GENERATION OF λ-DHBV RECOMBINANTS

In the examples detailed herein, we describe the generation, expression, and packaging of recombinant DHBV genomes containing heterologous gene sequences. A deletion was made in a region of the DHBV genome which encodes the surface antigen and viral polymerase proteins. Different sized fragments of bacteriophage λ genomic DNA were subsequently inserted into this region.

7.1. GENERAL PROCEDURES

7.1.1. CELLS

The human hepatoma cell line, HuH7 (Nakabayaski et al., 1982, Cancer Res. 42:3858-3863) was obtained from Dr. C Koiki (Department of Gene Research, Cancer

Institute, Tokyo, Japan) and was propagated in DMEM: F12 (1:1, v/v) (GIBCO, Grand Island, NY) medium plus 10% FBS.

7.1.2. PREPARATION, RESTRICTION AND MODIFICATION OF DNA

The DHBV sequences used in the construction of λ-DHBV recombinants were obtained from DHBV genomic DNA cloned into a plasmid pSP65 vector at the EcoRI site. λ genomic DNA was purchased from New England Biolabs (Beverly, MA). Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and the Klenow fragment of E. coli DNA Polymerase I were purchased from New England Biolabs (Beverly, MA). Reactions with these enzymes were carried out under conditions suggested by the supplier unless otherwise specified.

After restriction enzyme digestion, DNA fragments were separated and purified by gel electrophoresis in a 5% polyacrylamide gel in TBE buffer (0.01 M Tris-borate, pH 8.2, 1 mM EDTA). DNA fragments were visualized by negative shadowing over a PEI (polyethyline-imine) thin-layer chromatography plate under ultraviolet light. The DNA fragments were recovered by electroelution at room temperature for 2 hours at 200 V in TBE buffer.

7.2 CONSTRUCTION OF λ-DHBV RECOMBINANTS

λ-DHBV recombinants were generated by deleting sequences encoding the polymerase protein and surface antigen by digestion of the wild-type DHBV genome with KpnI. The linearized plasmid was filled in with the Klenow fragment of E. coli DNA Polymerase I. Fragments of bacteriophage λ were subsequently ligated into the plasmid (at nucleotide number 1290) with T4 DNA ligase. The λ-DHBV recombinants containing a 180 bp HaeIII λ fragment or a 1000 bp HaeIII λ fragment, were termed Kpn^-+180, and Kpn^-+1000, respectively.

Like the particle-defective DHBV genomes and the ΔDR1 mutant, the λ-DHBV recombinant was dimerized using either of the two strategies outlined in Section 6.2. The λ-DHBV recombinant cloned into SP65 was amplified by transformation into E. coli HB101 cells. The dimer clones were subsequently identified by restriction analysis of plasmid DNAs.

7.3. EXPRESSION OF RECOMBINANT λ-DHBV GENOMES

The recombinant λ-DHBV genomes were expressed by cotransfecting λ-DHBV genomes together with the packaging genome ΔDR1 into HuH7 cells using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973, Virology 52:456-467). The expression of the λ-DHBV recombinant was monitored by Southern blot hybridization using procedures described in Section 6.4.2. As shown in figure 3, lane 8, the λ-DHBV recombinant containing the 180 bp HaeIII fragment of λ (Kpn⁻+180) was packaged into virions, however, the packaging occurred at a level approximately 50-fold less than the Kpn⁻ mutant. The λ-DHBV recombinant containing the 1000 bp HaeIII fragment of λ (Kpn⁻+1000) was not packaged (see figure 3, lane 7). These results indicate that there is a size constraint for packaging of the genome and that, furthermore, in this system, there is apparently a strict limitation on the maximum size of the genome for packaging of approximately 3000 bp. In order to insert larger heterologous fragments, larger DHBV sequences should be deleted an/or DHBV DNA should be cloned into other vectors where there may be less of a size constraint.

8.0 GENERATION OF STABLE CELL LINES CAPABLE OF PRODUCING DEFECTIVE HEPADNAVIRUS

Transient expression systems are c nstrain d by inherent limitations, primarily the efficiency of

transfection. For this reason, it is desireable to incorporate one or more of the defective hepadnaviral genomes of the invention into permanent, stably-transfected cell lines. A stably transfected permanent cell line containing the correct genetic information is capable of substantially continuous production of viral particles.

Within the scope of this invention are permanent cell lines stably transfected with a hepadnaviral packaging genome, to produce a "packaging cell line". A packaging cell line is stably transfected with genetic information to provide complementation, in trans, of viral functions required by a second defective hepadnaviral genome transfected into the packaging cell line. More specifically, transfection of a particle defective genome into a packaging cell line results in supply of the helper functions required in trans by the "packaging" genome to enable the input genome to be packaged and reverse transcribed to produce infectious defective viral particles carrying the particle defective genome. The packaging genome, present within the packaging cell line, is not itself recovered in the form of infectious particles, since the packaging genome lacks the cis acting sequences necessary for its own replication into dsDNA.

Similarly, particle defective hepadnaviral genomes carrying a heterologous gene sequence (Section 5.2, supra) can be propagated and recovered in the form of infectious viral particles following introduction of the genome into a packaging cell line and complementation of the functions required by the particle defective genome.

The invention further relates to the production and use of permanent cell lin s stably containing more than one defective hepadnaviral genome, and the use of such cell lines as "producer"

cell lines for gen rating defective hepadnaviral particles. Specifically, a producer cell line may contain stably incorporated therein both a packaging genome and a particle defective genome. The producer cell line is capable of packaging and producing packaged particle defective genomes in a substantially continuous manner, and obviates the need for repeated transfections.

In the case of all cell lines capable of packaging and producing infectious defective genome particles, the particles are recoverable from the supernatant of cultured transfected cells using known methods.

8.1 PROCEDURES

8.1.1. CELLS

The HepG2 cell line is a human hepatoma cell line available in many laboratories. The HepG2 cells retain many morphological features which are characteristic of primary hepatocytes. The HepG2 line has previously been demonstrated to be capable of producing wild-type virus following transfection with wild-type HBV DNA in dimer form. See, e.g., Sells et al., Proc. Nat. Acad. Sci., U.S.A. 84:1005, 1987. Insofar as has been determined, cells of the HepG2 cell line contain no endogenous HBV DNA sequences.

8.1.2. PREPARATION OF A PLASMID VECTOR COMPRISING A PACKAGING GENOME DIMER AND A SELECTABLE MARKER

For transfection of the HepG2 cell line with the packaging genome ΔDR1, the plasmid vector designated pHBVΔDR1Neo (Figure 10) was constructed. The vector pHBVΔDR1Neo comprises a dimer form of the human HBVΔDR1 packaging genome, and further comprises

an antibiotic (neomycin) resistance gene und r control of an SV40 promoter.

A reproducible method for constructing pHBV DR1Neo using established laboratory procedures is illustrated schematically in Figure 11 and described as follows. A 479 base pair XmnI-BglI fragment carrying ampicillin resistance is excised from the E. coli plasmid pSP65, and the plasmid backbone filled in with the Klenow fragment of DNA polymerase I and blunt end ligated to a 2057 bp ClaI-XmI fragment carrying chloramphenicol resistance derived from pBR328. The resulting plasmid construct was used to transform E. coli strain HB101 to chloramphenicol resistance. The construct pSPCAM has no restriction sites for the restriction enzyme FspI, and carries a polylinker region derived from pSP65.

A Hinc-XbaI fragment in the polylinker region of pSPCAM was then replaced with a 266bp fragment corresponding to positions 1726 to 1992 of the wild-type HBV genome, which fragment was obtained as a DraI-XbaI fragment from wild-type HBV dimer plasmid. The resulting CAM^R construct was used to transform E. coli strain HB101 to chloramphenicol resistance. A portion of the 266 HBV sequence in the CAM^R construct, which portion contains the DR1 sequence at positions 1826 to 1837, inclusive, was excised as a FspI-StyI fragment, which fragment comprises nucleotide positions 1804 to 1884. The excised fragment was replaced with a homologous synthetic fragment in which nucleotides 1826 to 1837 were precisely deleted, and in which FspI and StyI sites were precisely regenerated for inserting the synthetic fragment into the plasmid construct in proper orientation. Removal of nucleotides 1826 to 1837 deletes the DR1 sequence within the construct.

The synthetic FspI-StyI fragment was prepar d by synthesizing complementary strands using

an applied Biosystems 280A synthesizer. The strands were blocked, gel-purified, phosphorylated and annealed using standard methods. To identify correct deletion fragments from the resulting clones, recombinants were probed in situ with a ^{32}P-labeled fragment which contained 10 bases from each side of the DR1 deletion joined together. Hybridization was carried out at reduced temperature (10°C below T melting), at which temperature the probe fails to hybridize to wild-type HBV. Positive clones carrying the correct deletion were then analyzed on a sequencing gel to confirm precise deletion of the DR1 sequence, thereby identifying Δ DR1 mutants.

Recombinant CAM^R constructs carrying the synthetic fragment were next reacted with the enzymes XbaI and Hind III to excise a large fragment containing the added HBV sequence including the Δ DR1 region, as well as additional construct sequences on the end of the fragment adjacent to the Hind III restriction site. The fragment was used to replace a Hind III - XbaI fragment in pSP65. Into the recombinant pSP65 vector was inserted an XbaI-EcoRI fragment from wild-type HBV dimer containing nucleotide positions 2143 to 3182, and an XbaI fragment from wild type HBV dimer containing nucleotide positions 1992-2143. Fragments were ligated in the orientations shown in Figure 11. Positive recombinants containing all fragments were identified by hybridization using corresponding labeled wild-type HBV probes, and orientation was confirmed by sequence analysis.

From the recombinant, a FspI-EcoRI fragment containing nucleotides from positions 1804 to 3182 was removed. The fragment was first ligated to a 5' fragment of HBV derived by Hind III-Fsp I digestion of wild-type HBV dimer plasmid, and then into a pBR322 backbone, in a trimolecular reaction as shown in

Figure 11. The pBR322 backbone was prepar d by removing an EcoRI-HindIII fragment from pBR322 to generate cohesive EcoRI and HindIII termini on the backbone. Recombinants from the trimolecular reaction containing correct 3' and 5' HBV sequences were identified using probes corresponding to both of those sequences. The ΔDR1 genome was then dimerized by partially digesting the recombinant monomer with EcoRI, and inserting a second EcoRI-EcoRI fragment, corresponding to a second ΔDR1 genome derived from another monomer. Dimer plasmids were identified by miniplasmid DNA preparation and sizing in agarose gels, and correct structural orientation was confirmed using restriction analysis.

From the complete dimer, a ClaI-SalI fragment was excised, which fragment lies outside of and does not interfere with the tandem ΔDR1 genomes in the dimer. The fragment is replaced with a ClaI-SalI fragment containing a bacterial neomycin resistance gene under the control of an SV40 promoter. Source plasmids wherein a bacterial antibiotic resistance gene is controlled by an SV40 promoter are available and well known. See, e.g. Southern and Berg, 1982, J. Mol. Applied Genet., 1:327.The resulting vector was designated pHBVΔDR1Neo.

Of course, other vectors may be prepared by available methods which comprise a defective viral genome dimer and a selectable marker. The vector pHBVΔDR1Neo is described herein as exemplary of a suitable vector.

8.2 PRODUCTION AND ANALYSIS OF A HUMAN PACKAGING CELL LINE

To illustrat generation of a packaging cell line in acc rdance with the invention, the p rmanent

human hepatoma cell line HepG2 was transfected with the plasmid pHBVΔDR1Neo.

Prior to transfection, cultured HepG2 cells were seeded at 2 x 10^6 cells in 10 centimeter dishes in Dulbecco's Modified Eagle's Medium (DMEM:F12) (Gibco, Grand Island, New York) plus 10% fetal calf serum. This medium is used for normal carriage of the HepG2 line. Medium was changed the morning prior to transfection, and transfection was carried out by the calcium phosphate precipitation method. Eight (8) micrograms of plasmid DNA was transfected into each of three 10 centimeter dishes, and the medium changed after 8 hours. Two days following transfection, the cells were trypsinized from confluent plates and seeded out at densities of 350,000 cells per plate, 175,000 cells per plate and 75,000 cells per plate into medium containing G418 (Gibco: Grand Island, New York) at a concentration of 750 micrograms per milliliter. Cells were refed with fresh drug-containing medium every 3-4 days for a period of three weeks, at the end of which time the only viable cells were present as discrete colonies. G418 was then omitted from the medium and the cells were fed for one additional week, at the end of which colonies were picked using cloning rings and incubated for several minutes at 37°C in PBS containing 5 mM EDTA. Cells were then transferred to 24-well microtiter dishes containing DMEM:F12 plus 10% fetal calf serum, and the cells fed every 3-4 days.

To screen the transfected cells for production of hepatitis B surface antigen, growth of the cells in the microtiter wells was continued until growth was sufficient to produce an acid-appearing medium, indicating proper cell density . The cell medium was then harvested for hepatitis B surface antigen assay of the medium and replaced with fresh medium. Hepatitis B surface antigen assay f th

medium was carried out using a commercially available solid phase radioimmunoassay kit (Connaught Laboratories) essentially following the manufacturer's instructions. Approximately 200 different microtiter wells of transfectants were analyzed by radioimmunoassay, and 5 positive clones were identified which registered counts two fold above background. These positive clones were then further assayed for production of hepatitis B "e" antigen (precore protein; "HBeAg").

Medium from the wells testing positive for hepatitis B surface antigen was assayed for HBeAg using an anti-eAg antibody sandwich assay (Abbott Laboratories Diagnostic Kit-Abbott Laboratories, Abbott Park, Ill.). Of the five clones which had registered positive for HBsAg, a single clone registered strongly positive for HBeAg production. This clone, designated HepBl-2, was expanded by recloning. Subsequent retesting of HepBl-2 for both HBsAg and HBeAg production tested strongly positive.

Analysis of the HepBl-2 cell line was conducted to further characterize the cell line, including immunofluorescence analysis, electron microscopy and nucleic acid analysis of the supernatant culture fluid.

Immunofluorescent staining analysis for HBsAg was carried out on fixed HepBl-2 cells. As a control, HepG2 cells were examined using the same procedure. In each instance, cells were fixed on glass cover slips using cold 95% ethanol/5% glacial acetic acid. The plates were left in the fix solution at -20°C for two days, then washed with phospate-buffered saline (PBS) and air dried. The cover slips were then incubated with a solution of goat polyclonal anti-HB surface antigen (DAKO, Santa Barbara, Cal.) for 2 hours at 37°C, washed in PBS, then incubated with fluorescein-conjugated rabbit

anti-goat antiserum (DAKO) for a further 2 hours at 37°C. Cells were then examined using epifluorescence optics. HepB1-2 cells exhibited a uniform pattern of cytoplasmic immunofluorescence in all cells tested, suggesting that all cells of the HepB1-2 clonal expansion were derived from the same original clone. Control HepG2 cells exhibited no detectable fluorescence.

Due to the presence of both HBsAg and HbeAg within the HepB1-2 cells, the cells were examined by electron microscopy for production of Dane particles. As seen in Figure 12, electron microscopy of a thin section of HepB1-2 cells reveals visible budding of enveloped particles from the surface of the cells. The particles are of correct diameter (approximately 40nm) to be Dane particles. Similar budding particles are not observed upon similar examination of parental HepG2 cells.

Supernatant fluids from cultures of HepB1-2 cells were examined for the presence of particles using density gradient centrifugation. To prepare the medium from HepB1-2 cells for analysis, 40 ml samples of medium from confluent cultures were combined with cesium chloride (1g/3ml) and placed in sealed-top gradient tubes, followed by centrifugation in a Ti50.2 rotor (Beckman Instruments) for 60 h at 49,000 rpm. Fractions (1.4 ml) were collected serially from the top of the tube and the density of each fraction, determined by refractive index, was measured using 20 microliter samples in a refractometer (Bausch and Lomb). Samples of each fraction were then assayed for HBsAg using radioimmunoassay. A peak of antigen was detected at a density of approximately 1.20 g/ml in the CsCl density gradient, which corresponds to the exp cted sedimentation density of Dane particles. Analysis of the 1.20 density fraction by electron microscopy, as describ d by Sells et al., 1987, Proc.

Nat. Acad. Sci., U.S.A. 84:1005, revealed th presence of enveloped particles substantially res mbling those budding from HepB1-2 cells, as shown in Figure 12. Particles were not observed in other, neighboring density fractions.

Southern blot hybridization analysis of HepB1-2 genomic DNA following restriction with EcoRI, SacI, HindIII and PstI indicates that the cells contain a single intact copy of the Δ DR1 HBV dimer sequence which has integrated into the chromosomal DNA. The configuration of the integrated plasmid into the cellular DNA sequences is shown in Figure 13.

The particles produced by the HepB1-2 cell line were analyzed by various methods to determine whether the particles contained infectious HBV DNA. It would be predicted that the particles would not contain infectious DNA since the HepB1-2 cell line contains only a packaging genome (ΔDR1) which lacks an essential cis-acting replication region. As indicated in Section 6.4, supra, transfection of a Δ DR1 packaging genome into HuH7 cells failed to produce relaxed circular DNA, but apparently produced minor but detectable quantities of single stranded (-) strand DNA, as shown in Figure 3, lane 5. The minor amount of detectable ssDNA may be due to anamolous initiation of first-strand DNA synthesis. It was anticipated that, due to the similar mutation in the DR1 repeat region present in the plasmid DNA used to prepare the packaging cell line HepB1-2, the particles produced by the packaging cell line would consist predominantly of pregenomic RNA species unable to complete replication of dsDNA.

To analyze the nucleic acid content of the particles present in the packaging cell line supernatant, the supernatant was fractionated in a cesium chloride gradient. Nucleic acids w r then isolated from the different fractions by treatment

with 15mM EDTA, 0.5% sodium dod cyl sulfate, and 200 microgram/ml Proteinase K (Boehringer Mannheim) at 51°C for 90 minutes. Fractions were then phenol extracted and ethanol precipitated. Different fractions were analyzed in blot hybridization assays using a nick translated wild-type HBV probe. As shown in Figure 14, 3.0 kilobase species corresponding in size to an HBV genome were not detected in any of the fractions tested. Weakly hybridizing species were detected in fractions 1.18 - 1.25 g/ml and 1.26 - 1.32 g/ml of approximate 3.5 kilobase and 5 kilobase size, and a large smear that extends from 3.5 kilobases downward was observed in the density fraction 1.33-1.42 g/ml. The precise nature of these species has yet to be established, and their analysis is complicated by their low abundance. They most likely represent reverse transcription products of ΔDR1 RNA transcribed in the HepB1-2 cells, and as such would contain unusual sites for initiation of reverse transcription. They are almost certainly defective.

8.3 THE PACKAGING CELL LINE SUPPLIES FUNCTIONS IN TRANS TO COMPLEMENT A PARTICLE DEFECTIVE GENOME TRANSFECTED INTO THE PACKAGING CELL LINE

Based on the nature of the ΔDR1 mutation and the capability of the HepB1-2 cell line to produce detectable cytoplasmic viral antigens (Section 8.2, supra), it was expected that the cell line would be capable of providing packaging functions in trans to both package and reverse transcribe the RNA from a second defective HBV genome introduced into the cells. For example, the packaging cell line should fully complement in trans all viral functions required by a particle-defective HBV genome. Transfection of th particle-defective genome into the packaging cell line

should result in production of a packaged particl -defective genome which is recov rable in the cell supernatant.

For purposes of transfecting the HepBl-2 packaging cell line, an additional particle defective dimer genome was generated. The defective genome, designated X-, was produced by restricting wild-type HBV genome with XhoI to cleave the genome at nucleotide position 129. The cohesive ends generated by XhoI digestion were filled in with Klenow fragment, and a 10 bp synthetic linker containing a SalI restriction site was blunt end ligated into the XhoI site. The resulting X- defective genome was dimerized into a pSP65 plasmid backbone, as described previously.

HepG2 cells were then transfected with the X- dimer plasmid using two different procedures. In the first procedure, using calcium phosphate precipitation, cells were seeded the night prior to transfection, and transfected with 20 micrograms of plasmid DNA. Medium was collected from the transfected cells for analysis 3, 4, and 5 days following transfection.

For the second procedure, involving lipofection, cells were seeded at low density and allowed to grow for several days until nearly confluent. Lipofectin (Bethesda Research Laboratories: Gaithersburg, MD) was used to introduce an average of 5 micrograms of plasmid DNA, and in a different trial, 1 microgram of plasmid DNA, into the cells as follows. Plasmid DNA was mixed with 150 microliters of water in a polystyrene tube. 150 microliters of Lipofectin was then added and the tube was gently shaken to mix the DNA. Recipient cells were washed twice with DMEM (less serum). To each dish was added 9 ml Optim m (Bethesda Research Laboratories, Gaithersburg, MD) containing

mercaptoethanol. After 15 minutes at room temperature, the DNA mixture, which was then mildly cloudly, was applied dropwise to the cells. The DNA mixture was left on the cells for 24 hours, after which period the cells were washed 5 times with PBS, then cultured in DMEM plus 10% fetal calf serum. The cells were again washed 5 times with PBS the next day, and after an additional day, were washed 3 more times with PBS. The medium was then collected on days 4, 5, and 6 following transfection, and the recovered medium pooled at 4°C.

Medium recovered from the supernatants of transfected HepB1-2 cells was fractionated on a cesium chloride gradient and the nucleic acids isolated, as described previously. Nucleic acids were analyzed using Southern blot hybridization employing a random nick-translated HBV probe.

Figure 15 shows the results of Southern blot hybridization of supernatants from two transfections of HepB1-2 cells with X- dimer plasmid. In one transfection, 5 micrograms of plasmid DNA were employed, and in the second transfection, one microgram of plasmid DNA was employed. Supernatants from the transfectant cultures were fractionated in cesium chloride and each fraction was treated to isolate nucleic acids as described in Section 8.2, supra. Figure 15 reveals that, in both transfection supernatants, in the 1.20 g/ml density fraction, a 3.0 kilobase hybridizable species is present, which corresponds to the expected size of an HBV genome carried within a Dane particle. The observed species migrating slightly faster than the 3.0 kilobase fragment may represent closed circular forms of the HBV genome. Additional species which are observed at 9 kilobase size and larger r present residual input plasmid DNA. These larger species are observed

throughout the density gradient, and restriction analysis confirms their identity.

To establish the identity of the 3.0 kb species, the 3.0 kb species were excised from a low melting point agarose gel (Seakem, FMC) and following melting of the gel slice at 65°C for 4 minutes, digested with the enzymes SalI and NcoI at 37°C for two hours in the presence of 1 microgram of carrier mouse DNA. The digestion products were then analyzed in Southern blot analysis with a nick-translated HBV probe. Precisely as expected from a packaged mutant X^- genome, hybridizing fragments of 1900 bp and 1200 bp were observed.

In accordance with the invention, producer cell lines may be prepared by transfecting permanent cell lines such as HepG2 with more than one defective hepadnaviral genome. For example, if the packaging cell line HepB1-2 is further stably transfected with a particle defective genome, like X^-, the resulting cell line may constitute a permanent producer of the packaged form of the defective genome. In this manner, every cell in the transfected population, as opposed to the low percentage of cotransfectants available in the context of a transient expression system, becomes a producer of the particle defective genome. In this manner, greater titers of defective hepadnaviral particles could be recovered without repeated transfection steps.

9. DEPOSIT OF MICROORGANISMS

The following E. coli carrying the listed plasmids were deposited on July 12, 1988 with the Agricultural Research Culture Collection (NRRL), Peoria, IL and have been assigned the following accession numbers:

E. coli	Plasmid	Accession Number
HB101	pSP65 DHBV 5.2 Galx2 (wild-type DHBV)	B-18383
HB101	pSP65 DHBV RV$^-$265x2 (core DHBV)	B-18384
HB101	pSP65 DHBV Kpn$^-$Sph$^-$x2 (pol$^-$env$^-$core$^-$ DHBV)	B-18385
HB101	pSP65 DHBV DR1x2 (DR1$^-$ DHBV)	B-18386
HB101	pHBVΔDR1Neo	NRRL B-18529

The producer cell line designated HepB1-2 has been deposited with the American type Culture Collection, and assigned Accession Number ATCC CRL 10190.

The present invention is not to be limited in scope by the microorganisms deposited since the deposited embodiments are intended as single illustrations of one aspect of the invention and any microorganisms or viruses which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purpose of description.

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International Application No: PCT/US89 / 03521

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 88, line 11 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]

Name of depositary institution [4]

Agricultural Research Service Culture Collection (NRRL)

Address of depositary institution (including postal code and country) [4]

1815 North University Street
Peoria, Illinois 61604, United States of America

Date of deposit [5]	Accession Number [6]
3 August 1989 (03.08.89)	B-18529

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [✓] This sheet was received with the International application when filed (to be checked by the receiving Office)

Catherine E. W. Abrams
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

WEB

(Authorized Officer)

(January 1985)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ US89 /03521

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 88, line 12-15 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]

Name of depositary institution [4]

American Type Culture Collection (ATCC)

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852, United States of America

Date of deposit [5]	Accession Number [6]
2 August 1989 (02.08.89)	ATCC CRL 10190

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [✓] This sheet was received with the international application when filed (to be checked by the receiving Office)

Catherine B. Williams
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

(January 1985)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A DNA sequence that is incapable of supplying all hepadnaviral functions reqiured for hepadnaviral replication, which DNA sequence can be transcribed into an RNA capable of being (a) packaged into a hepadnavirus virion, and (b) reverse transcribed into DNA.

2. The DNA sequence of claim 1 comprising a mutation in the hepadnaviral gene selected from the group consisting of pol, env, and core.

3. The DNA sequence of claim 2 comprising mutations in pol, env, and core.

4. The DNA sequence of claim 2 comprising mutations in pol and env.

5. The DNA sequence of claim 2 in which the mutation comprises a deletion.

6. The DNA sequence of claim 2 which encodes a truncated hepadnaviral surface antigen.

7. The DNA sequence of claim 1 comprising a heterologous gene sequence.

8. The DNA sequence of claim 2 further comprising a heterologous gene sequence.

9. The DNA sequence of claim 5 further comprising a heterologous gene sequence.

10. The DNA sequence of claim 7 in which the heterologous gene sequence comprises a sequence ncoding an immunogenic pitope.

11. The DNA sequence of claim 8 in which the heterologous gene sequence comprises a sequence encoding an immunogenic epitope.

12. The DNA sequence of claim 9 in which the heterologous gene sequence comprises a sequence encoding an immunogenic epitope.

13. The DNA sequence of claim 7, 8 or 9 in which the heterologous gene sequence comprises a sequence encoding an enzyme.

14. A nucleic acid vector comprising the DNA sequence of claim 1.

15. A nucleic acid vector comprising the DNA sequence of claim 2.

16. A nucleic acid vector comprising the DNA sequence of claim 7.

17. A nucleic acid vector comprising the DNA sequence of claim 10.

18. A cell comprising the nucleic acid vector of claim 14, 15, 16 or 17.

19. A DNA sequence which

(a) can be transcribed into an RNA, which RNA is incapable of being (i) packaged into a virion, or (ii) reverse transcribed into a double-stranded hepadnaviral genomic DNA; and

(b) can be transcribed into a messenger RNA which encodes one or more proteins which can supply an in trans function necessary for (i) packaging a second RNA into a hepadnavirus

virion, or (ii) reverse transcription of the second RNA into DNA.

20. The DNA sequence of claim 19 comprising a mutation in a direct repeat region selected from the group consisting of DR1 and DR2.

21. The DNA sequence of claim 19 comprising mutations in DR1 and DR2.

22. The DNA sequence of claim 20 in which the mutation comprises a deletion.

23. The DNA sequence of claim 21 in which the mutation comprises a deletion.

24. A nucleic acid vector comprising the DNA sequence of claim 19.

25. A nucleic acid vector comprising the DNA sequence of claim 20.

26. A cell comprising the nucleic acid vector of claim 24 or 25.

27. A replication-defective hepadnavirus comprising a hepadnavirus that is capable of replication only in the presence of hepadnaviral protein other than that produced by expression of its genomic DNA, which genomic DNA can be transcribed into an RNA capable of being (a) packaged into a virion, and (b) reverse transcribed into DNA.

28. The hepadnavirus of claim 27 in which the genomic DNA comprises a mutation in the gene selected from the group consisting of pol, env, and core.

29. The hepadnavirus of claim 28 in which the genomic DNA comprises mutations in pol, env and core.

30. The hepadnavirus of claim 28 in which the genomic DNA comprises mutations in pol and env.

31. The hepadnavirus of claim 28 in which the mutation comprises a deletion.

32. The hepadnavirus of claim 28 which produces a truncated surface antigen.

33. The hepadnavirus of claim 28 which produces a truncated core antigen.

34. The hepadnavirus of claim 27 in which the genomic DNA comprises a heterologous gene sequence.

35. The hepadnavirus of claim 28 in which the genomic DNA further comprises a heterologous gene sequence.

36. The hepadnavirus of claim 31 in which the genomic DNA further comprises a heterologous gene sequence.

37. The hepadnavirus of claim 34 in which the heterologous gene sequence comprises a sequence encoding an immunogenic epitope.

38. The hepadnavirus of claim 35 in which the heterologous gene sequence comprises a sequence encoding an immunogenic epitope.

39. The hepadnavirus of claim 36 in which the heterologous gene sequence comprises a sequence encoding an immunogenic epitope.

40. The hepadnavirus of claim 34, 35, or 36 in which the heterologous gene sequence comprises a sequence encoding an enzyme.

41. The genomic DNA of claims 27, 28 or 31.

42. The genomic DNA of claim 34.

43. The genomic DNA of claim 36.

44. A replication-defective hepadnavirus which is produced by coexpression of the DNA sequence of claim 1 and the DNA sequence of claim 19 in a cell.

45. A replication-defective hepadnavirus which is produced by coexpression of the DNA sequence of claim 2 and the DNA sequence of claim 19 in a cell.

46. A replication-defective hepadnavirus which is produced by coexpression of the DNA sequence of claim 1 and the DNA sequence of claim 20 in a cell.

47. A replication-defective hepadnavirus which is produced by coexpression of the DNA sequence of claim 2 and the DNA sequence of claim 20 in a cell.

48. A replication-defective hepadnavirus which is produced by coexpression of the DNA sequence of claim 7 and the DNA sequence of claim 19 in a cell.

49. A replication-defective hepadnavirus which is produced by coexpression of the DNA sequence of claim 8 and the DNA sequence of claim 19 in a cell.

50. A c ll comprising the DNA sequence of claim 19.

51. A method of treating a patient with hepadnavirus infection comprising administering the hepadnavirus of claim 27 to the patient.

52. A method of treating a patient with hepadnavirus infection comprising administering the hepadnavirus of claim 28 to the patient.

53. A method of treating a patient with hepadnavirus infection comprising administering the hepadnavirus of claim 30 to the patient.

54. A method of treating a patient with hepadnavirus infection comprising administering the hepadnavirus of claim 32 to the patient.

55. A method of treating a patient with hepadnavirus infection comprising administering the hepadnavirus of claim 33 to the patient.

56. A permanent hepatoma cell line characterized by the substantially continuous production of defective hepadnavirus particles.

57. A cell line in accordance with claim 56 wherein said hepatoma cell line is a human cell line.

58. A cell line in accordance with claim 56 prepared by stably introducing at least one defective hepadnaviral genome into said permanent hepatoma cell line.

59. A cell line in accordance with claim 58 wherein the defective hepadnaviral genome is contained within a vector and the vector is used to transfect said permanent cell line.

60. A DNA vector comprising a defectiv hepadnaviral genome and a selectable marker.

61. A DNA vector comprising the DNA sequence of claim 1, 19 or 27 and a selectable marker.

62. The DNA vector pHBV DR1Neo.

63. A permanent hepatoma cell line characterized by the stable intracellular presence of the DNA sequence of claim 19.

64. The permanent hepatoma cell line of claim 63, further characterized by the capability of the cells to transcribe said DNA sequence into RNA and to express in trans function necessary for (i) packaging a second RNA into a hepadnavirua virion; or (ii) reverse transcribing a second RNA into DNA.

65. The human hepatoma cell line HepB1-2.

66. The cell line of claim 64 or 65 further comprising a second defective hepadnaviral genome.

67. The cell line of claim 66 wherein said second defective hepadnaviral genome is a particle-defective genome.

FIG. 1

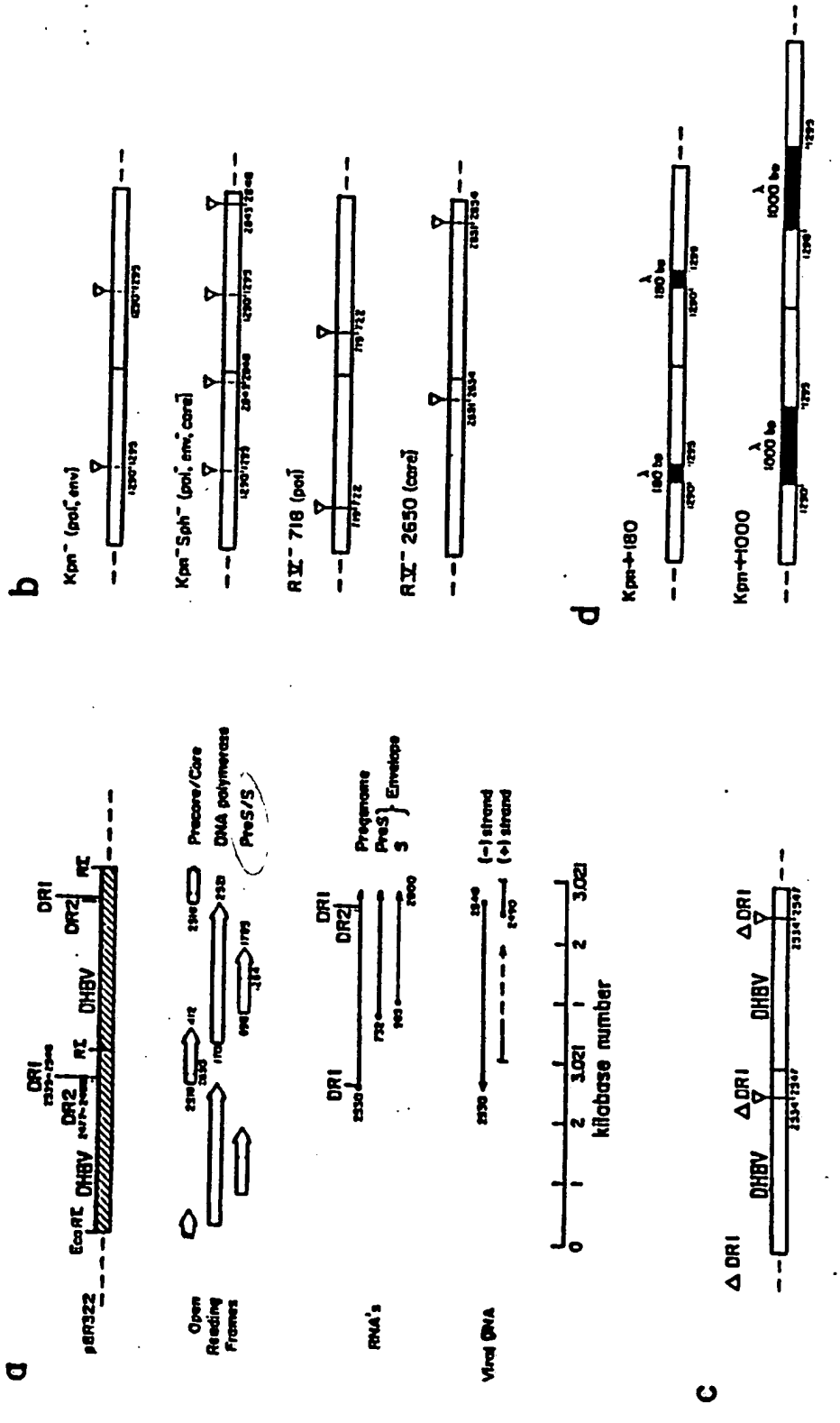
a
pBR322
EcoRI
DHBV
RI
DR2
DR1
RC
Open Reading Frames
Precore/Core
DNA polymerase
PreS/S
RNA's
Pregenome
PreS1
PreS2
S
Envelope
Viral DNA
(-) strand
(+) strand
0
1
2
3.021
kilobase number
b
Kpn⁻ (pol, env)
Kpn⁻Sph⁻ (pol, env, core)
RI⁻ 718 (pol)
RI⁻ 2650 (core)
c
Δ DR1
DHBV
Δ DR1
DHBV
Δ DR1
d
Kpn+180
Kpn+1000
180 bp
1000 bp

FIG 2

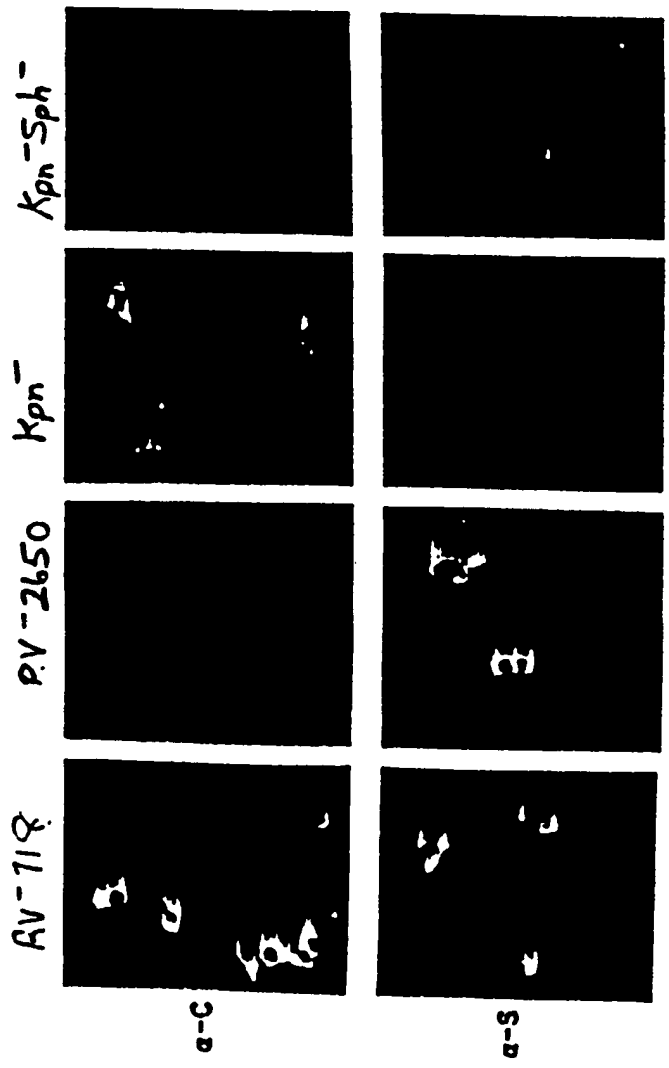

FIG. 3

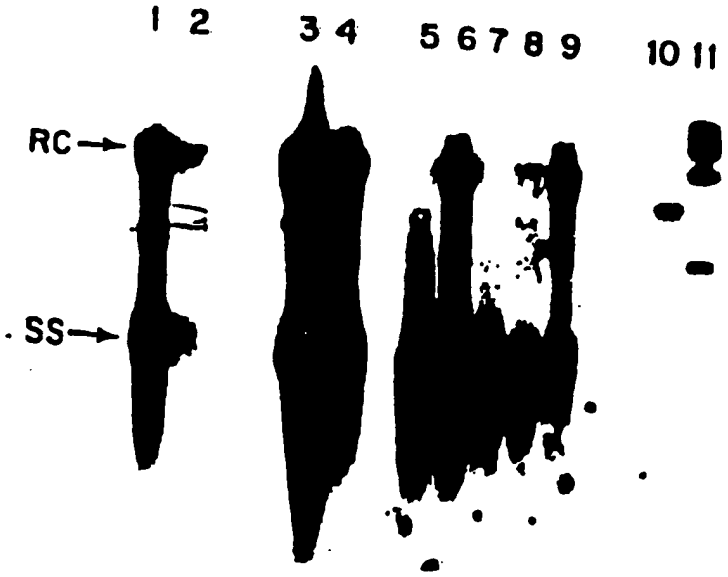

FIG. 4

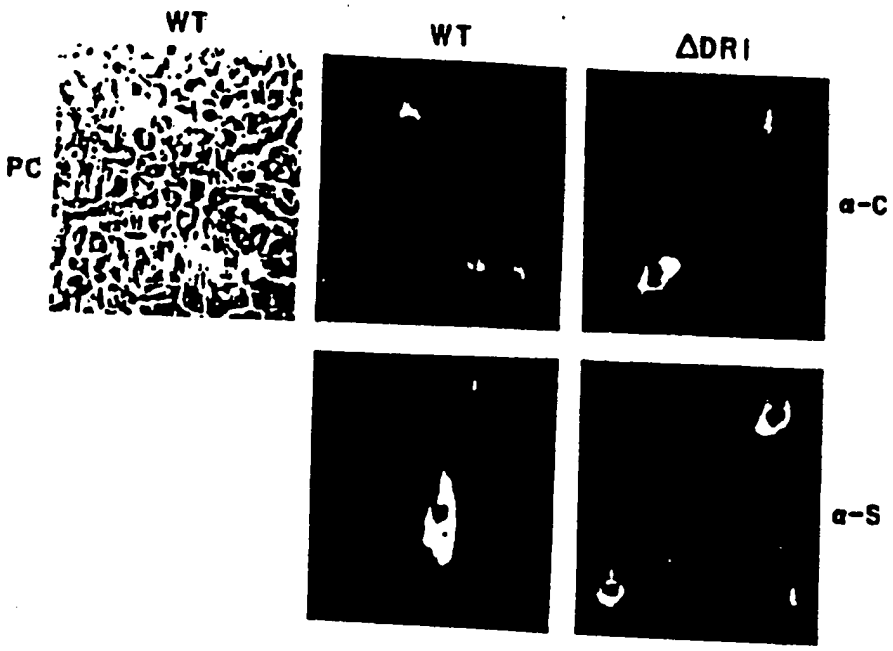

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FIG. 5

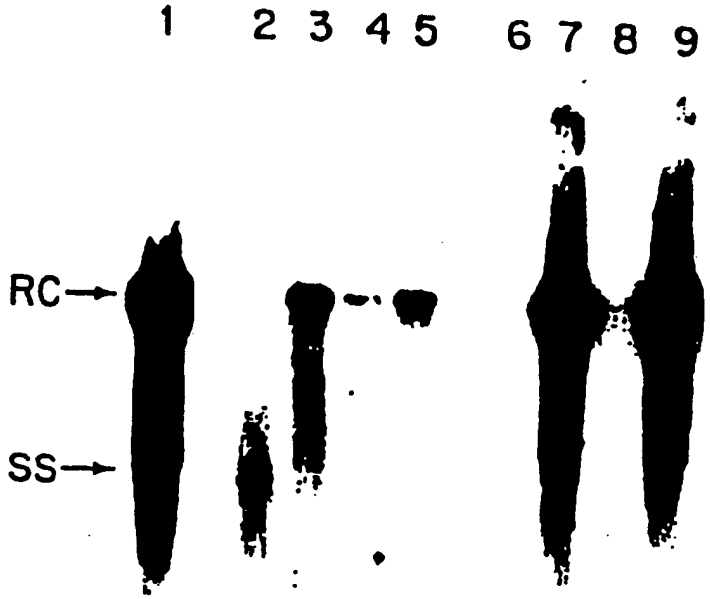

FIG. 6

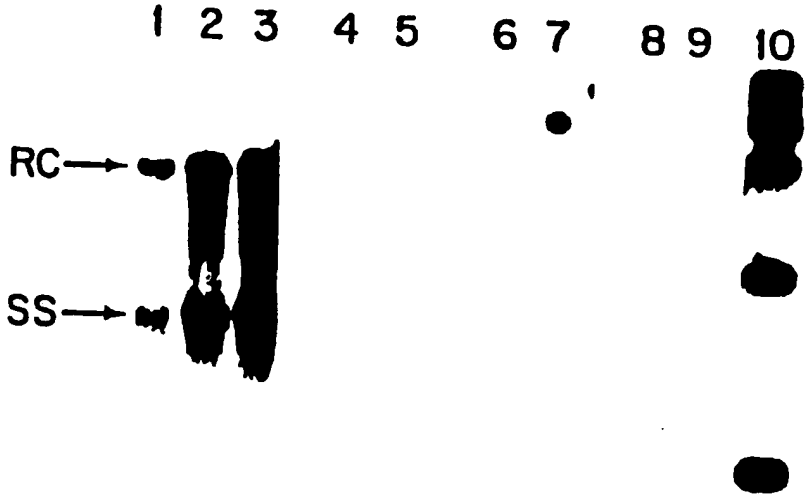

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FIG. 7

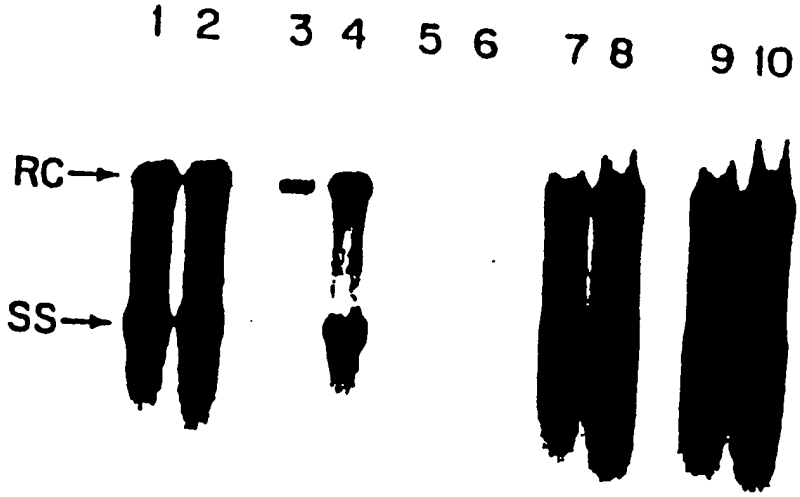
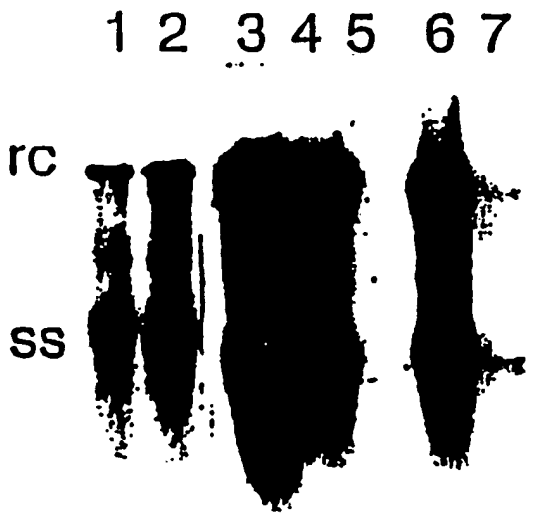


FIGURE 8A

[illegible]TTA CTG GTA AGC TTT TTC TTG TTG[illegible] WT

TAA CTG GTA AGC TTT TTC TAG TAG IS

FIGURE 8B



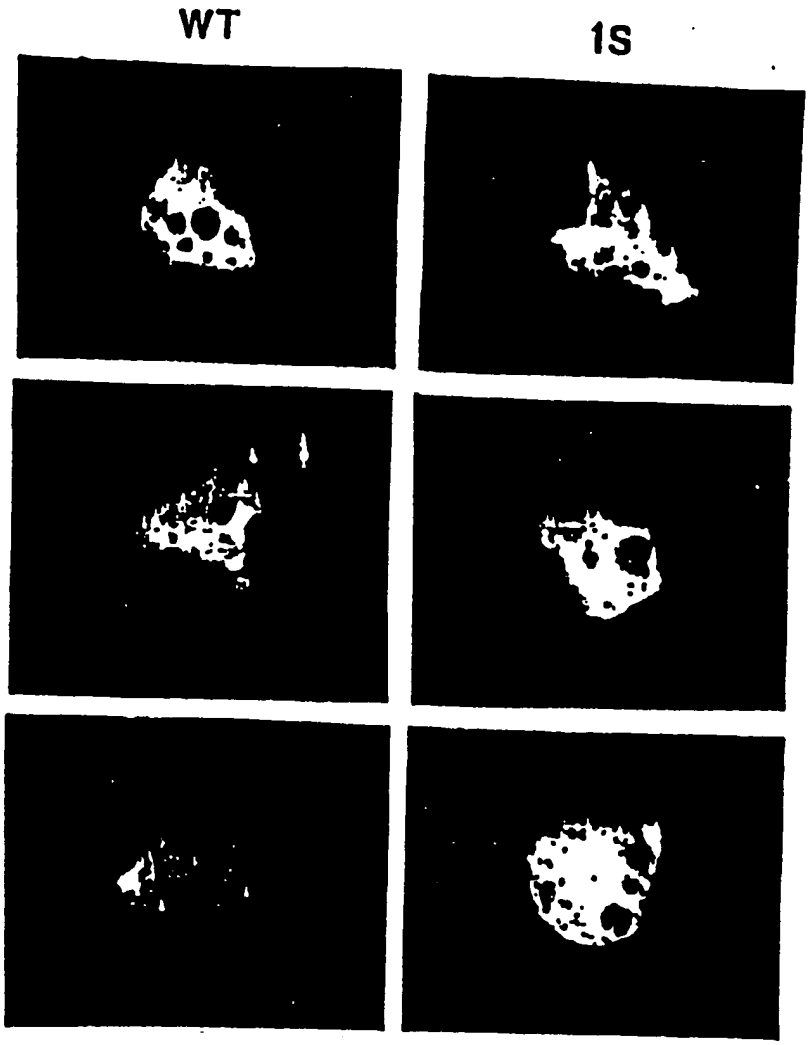


FIGURE 9

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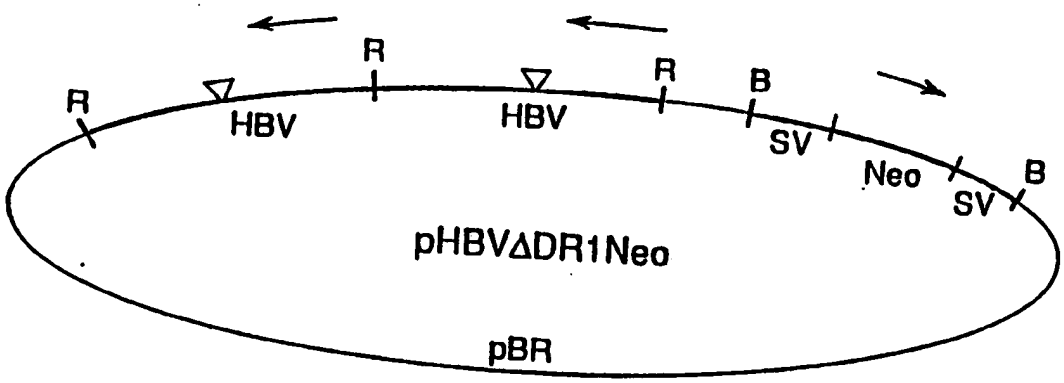

FIGURE 10

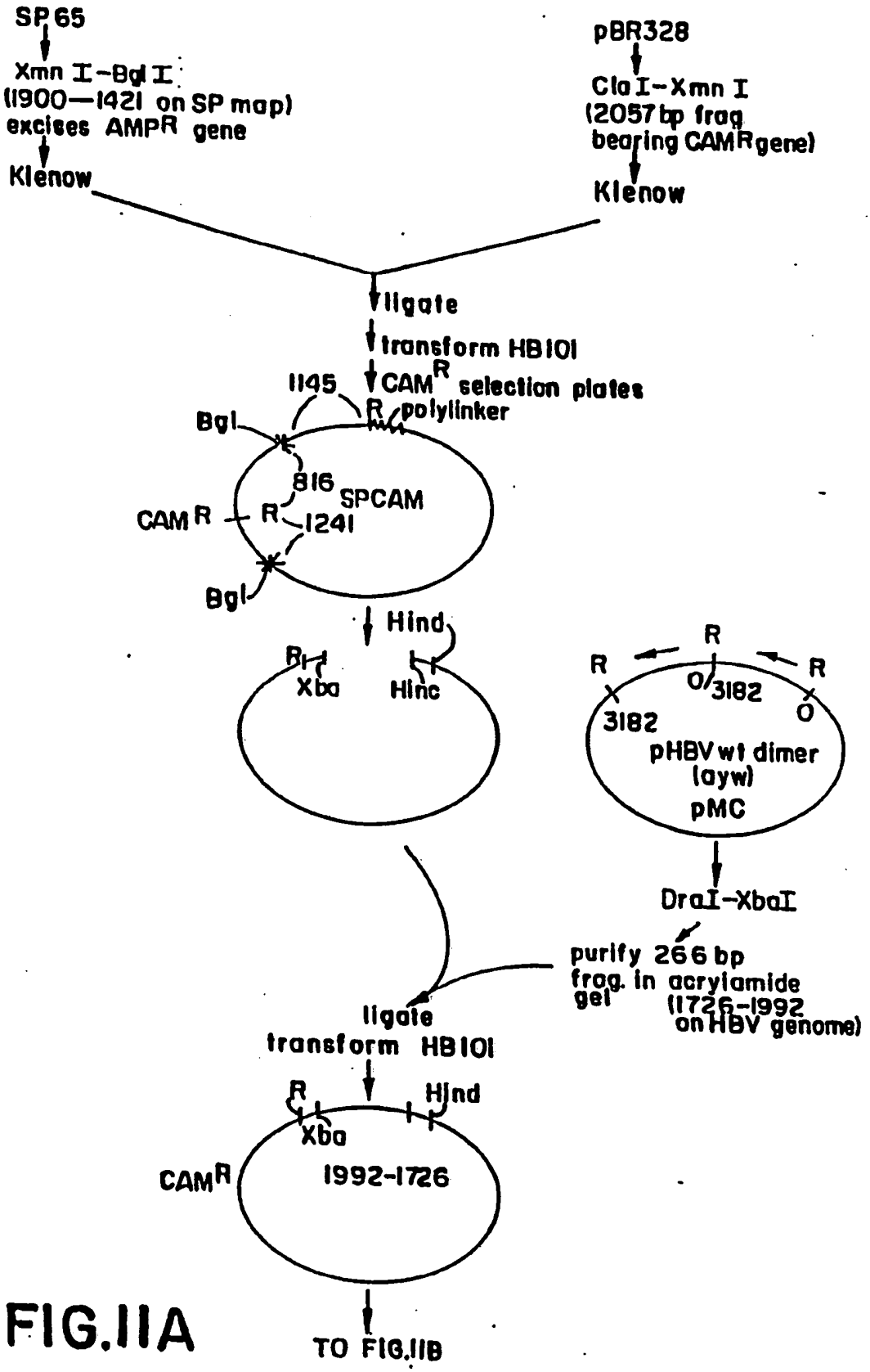
SP65
Xmn I-Bgl I
(1900—1421 on SP map)
excises AMP^R gene
Klenow
pBR328
Cla I-Xmn I
(2057 bp frag
bearing CAM^R gene)
Klenow
ligate
transform HB101
CAM^R selection plates
1145
R
polylinker
Bgl
816
SPCAM
CAM^R
R
1241
Bgl
Hind
R
Xba
Hinc
R
R
R
O/3182
3182
O
pHBV wt dimer
(ayw)
pMC
DraI-XbaI
purify 266 bp
frag. in acrylamide
gel
(1726-1992
on HBV genome)
ligate
transform HB101
R
Hind
Xba
CAM^R
1992-1726
TO FIG.IIB

FIG.IIA

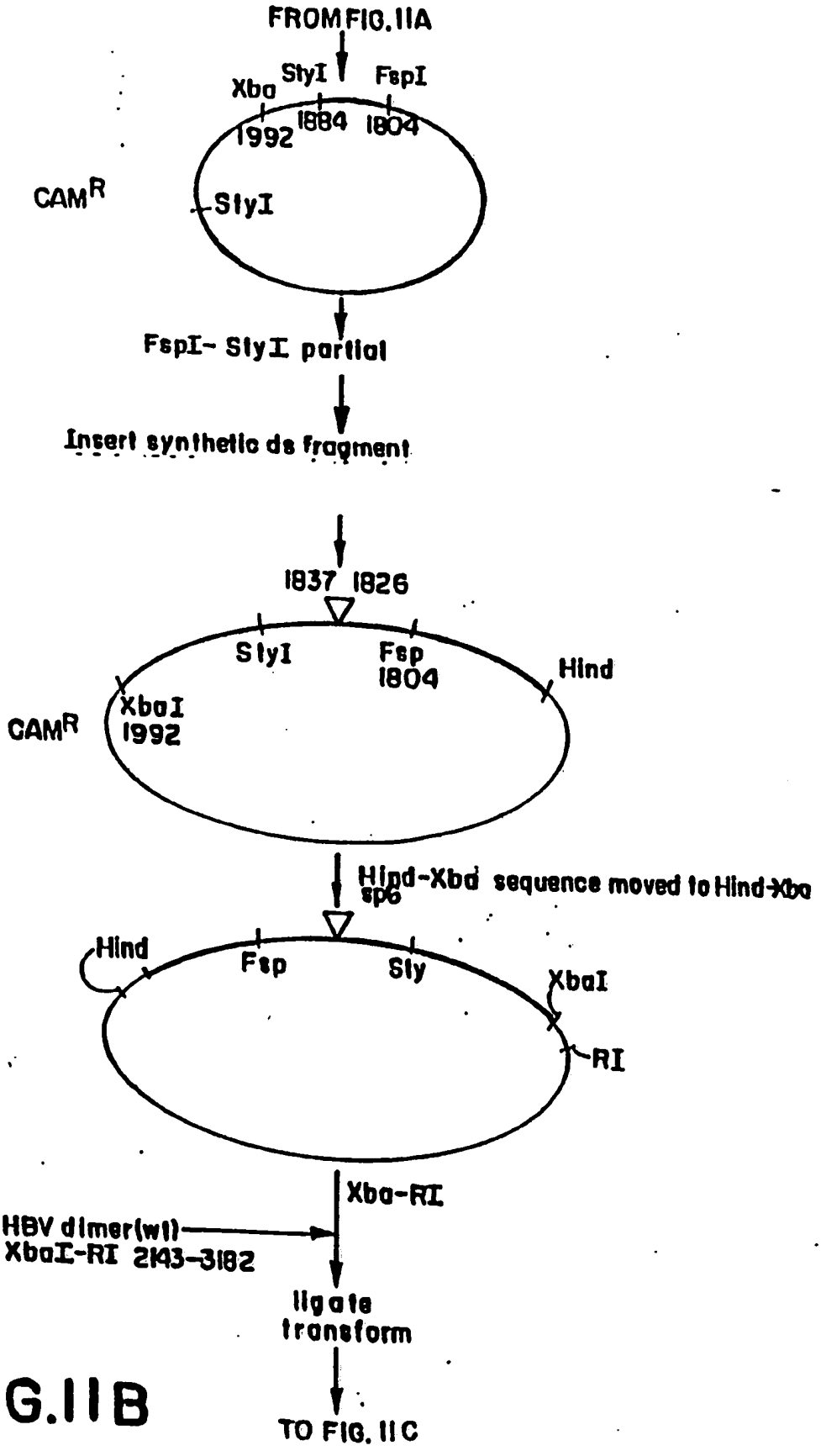
FROM FIG. IIA
StyI
FspI
Xba
1992
1884
1804
CAM^R
StyI
FspI- StyI partial
Insert synthetic ds fragment
1837 1826
StyI
Fsp
1804
Hind
CAM^R
XbaI
1992
Hind-Xba sequence moved to Hind→Xba
spc
Hind
Fsp
Sty
XbaI
RI
Xba-RI
HBV dimer(wt)
XbaI-RI 2143-3182
ligate
transform
TO FIG. IIC

FIG. IIB

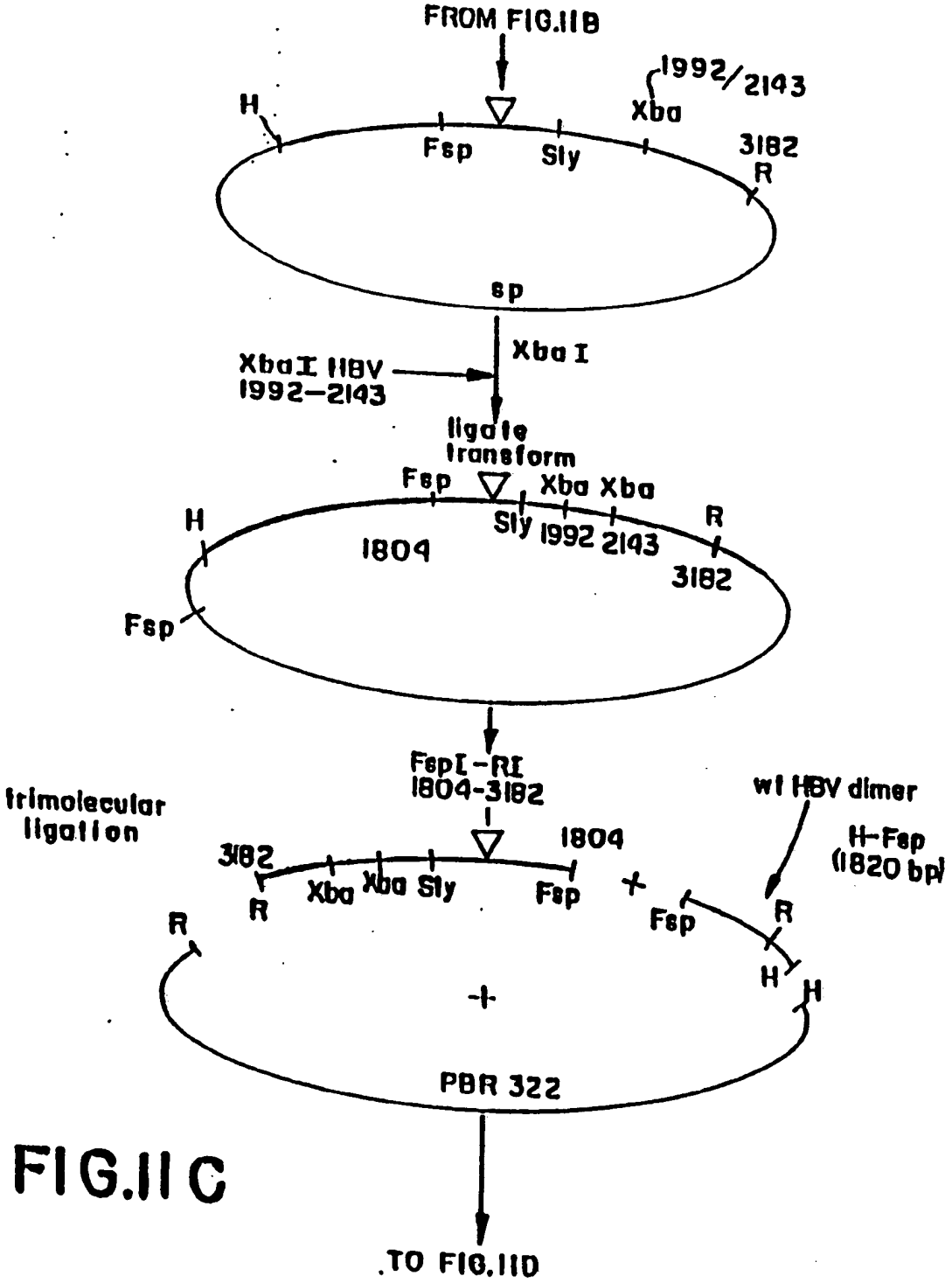
FROM FIG.IIB
1992/2143
H
Xba
Fsp
Sly
3182
R
sp
Xba I
Xba I HBV
1992–2143
ligate
transform
Fsp
Xba Xba
H
Sly
R
1804
1992 2143
3182
Fsp
FspI-RI
1804-3182
trimolecular
ligation
wt HBV dimer
H-Fsp
(1820 bp)
1804
3182
Xba
Xba
Sly
Fsp
R
R
Fsp
R
H
H
PBR 322
TO FIG.IID

FIG.II C

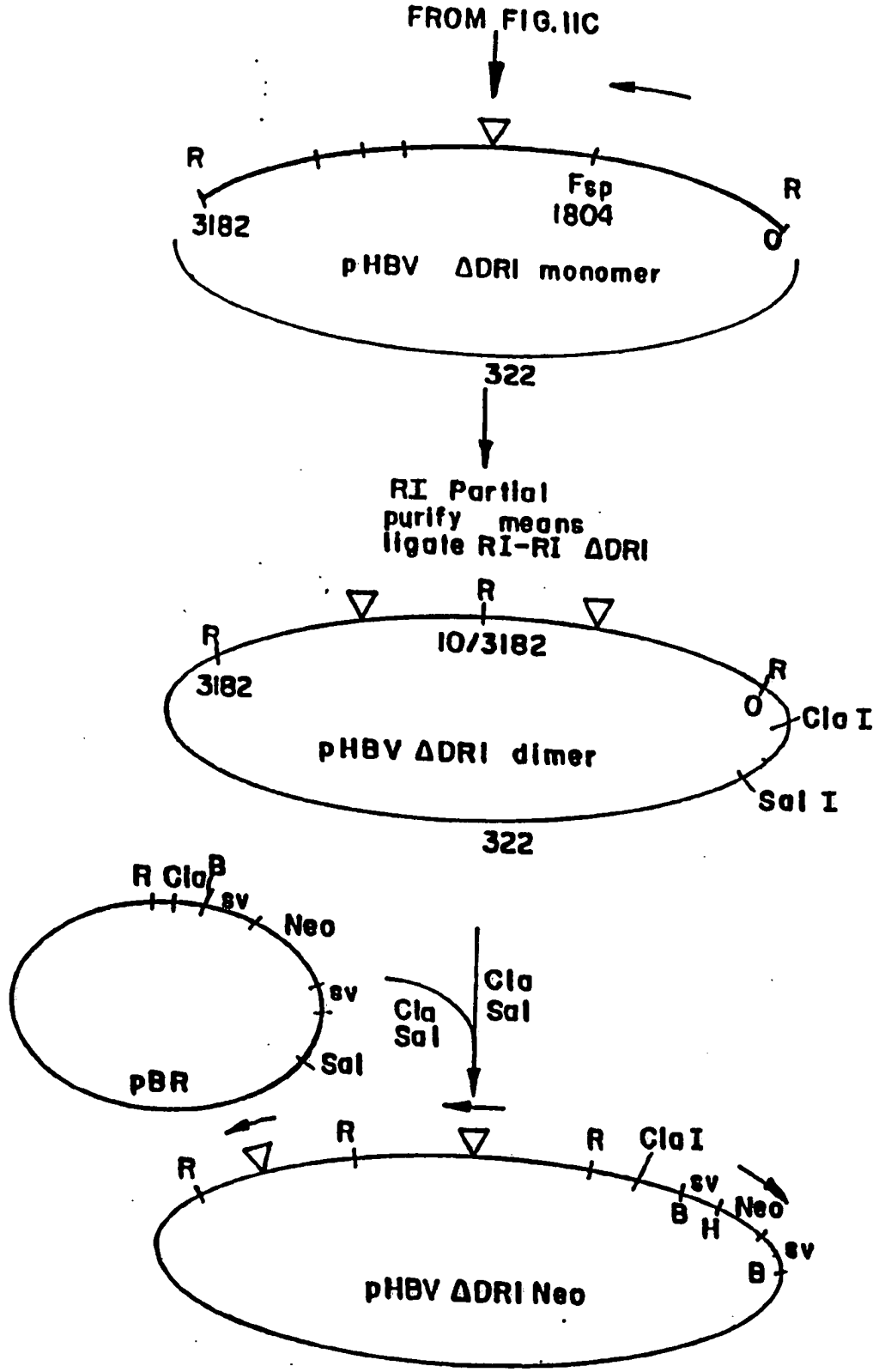
FROM FIG. IIC
R
3182
Fsp
1804
R
0
pHBV ΔDRI monomer
322
RI Partial
purify means
ligate RI-RI ΔDRI
R
10/3182
R
3182
R
0
Cla I
pHBV ΔDRI dimer
Sal I
322
R Cla B
sv
Neo
sv
Cla
Sal
Cla
Sal
Sal
pBR
R
R
R
Cla I
R
sv
B
H
Neo
sv
B
pHBV ΔDRI Neo

FIG. IID

FIGURE 12

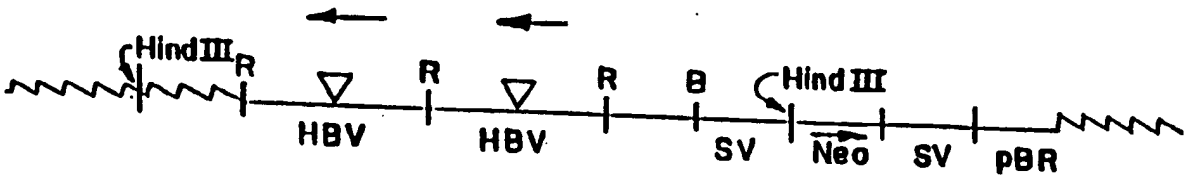

FIG. 13

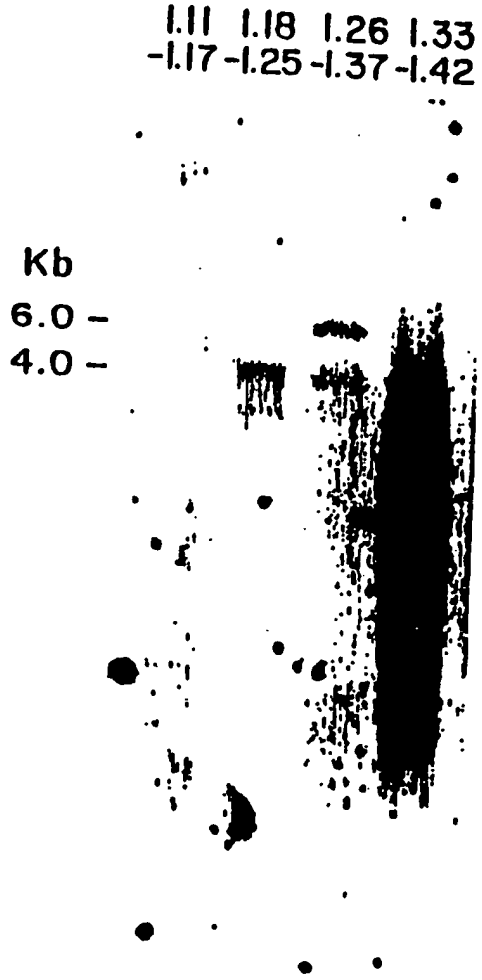

FIGURE 14

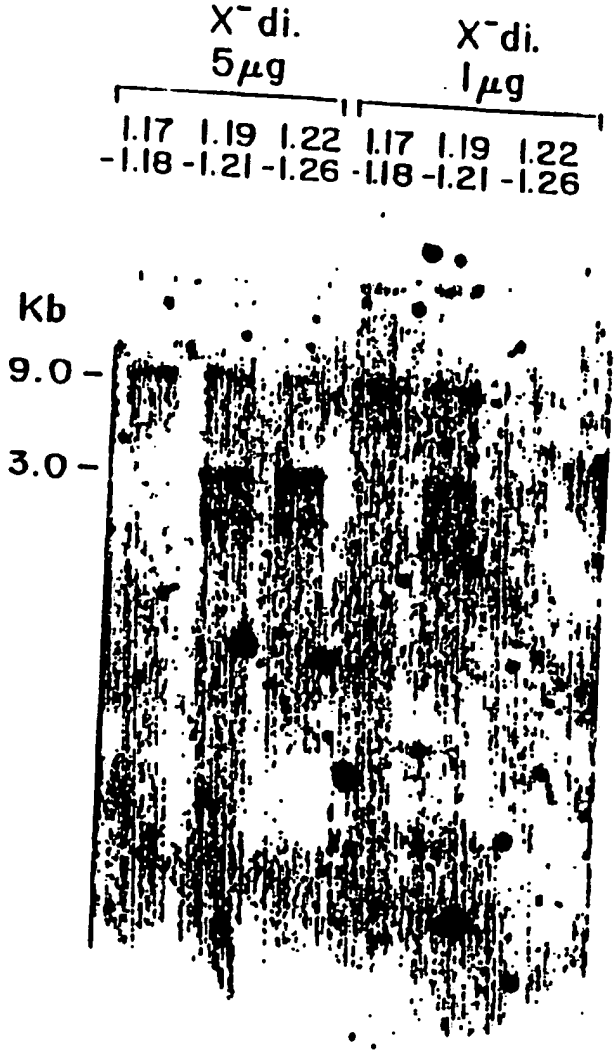
X⁻ di.
5μg
X⁻ di.
1μg
1.17 -1.18
1.19 -1.21
1.22 -1.26
1.17 -1.18
1.19 -1.21
1.22 -1.26
Kb
9.0 –
3.0 –

FIGURE 15

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03521

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00, 15/86; A61K 39/29; 35/76

U.S.CL.:435/172.3 , 424/95; 424/89

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/172.3, 235, 240.2, 320; 514/47 536/27; 424/89, 95

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Dialog "biotech" database, ChemAbstracts database and APS database; Keywords: hepatitus B virus, vector, DNA,ENV, P.1 gag antigen,vaccine, hepadnavirus, hepatitus

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
T	US, A, 4,861,588 (Neurath) 29 August 1989 See col. 2.	1-55
Y	Biological Abstracts, Vol. 84, No. 6, issued 15 September 1987, Tagawa, "Duckhepatitus B virus replicates in the yolk sac of developing embryos", see page 572, col. 2, the abstract no. 57385. J. Virol 61(7): 2273-2279. 1987.	1-55
X Y	Biological Abstracts, Vol. 78, No. 10, issued 15 November 1984, Seeger, "Nucleotide sequence of an infectious molecularly cloned genome of ground squirrel hepatitus virus", see abstract No. 75298, J. Virol. 51(2): 367-375. 1984.	1-13 27,28,31 34,36, 41-43, 14-26, 29-30, 32-33,35, 37-40, 44-55

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 January 1990	23 JAN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	THOMAS G. WISEMAN

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X/Y	Biological Abstracts, Vol. 81, No. 7, 01 April 1986, Schaeffer, "Indentification and localization of pre-s-encoded polypeptides from wood chuck and ground squirrel hepatitus viruses.," see Abstract No. 68488, J. Virol. 57(1): 173-182 1985.	1-13/14-55
X/Y	Proc. Natl. Acad. Sci. USA, Vol. 77, No. 4, issued April 1980, I.W. Cummings, "Isolation, characterization and comparison of recombinant DNAs derived from genomes of human hepatitus B virus and woodchuck hepatitus virus", see pp. 1842-1846	1-13/14-55
X/Y	Gene, Vol. 50., issued 1986, Jeanne Etiemble, "Nucleotide sequence of the woodchuck hepatitus virus surface antigen mRNAs and variability of three overlapping viral genes", See pp. 207-214.	1-13/14-55